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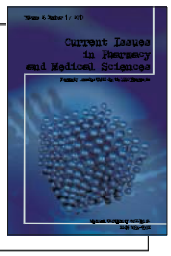

Examination of salivary proteins as biomarkers of pathological conditions. Literature review

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ABSTRACT

Saliva is universally available biofluid, easy to collect. Comprehensive analysis and identification of the proteomic content of human saliva may contribute to the understanding of oral pathophysiology and provide a foundation for the recognition of potential biomarkers of human disease. These features make it an ideal biological material for the early detection of many diseases of different origin, and enable non-invasive diagnostics. The presence of protein markers in saliva was found with usage of capillary electrophoresis and mass spectrometry..

Keywords: salivary proteins, salivary diagnostics, chromatography, mass spectrometry

INTRODUCTION

Human saliva is a fluid having many biological functions essential for the maintenance of oral health. Scientists have been more interested in the possible salivary role as an indicator of systemic or oral disease. The recent use of saliva in the diagnosis of human immunodeficiency virus (HIV), carcinoma in a number of tissues, cardiac disease and autoimmune diseases has demonstrated that saliva can be a useful aid to clinical diagnosis [1,3,7,11,18,21].

Whole saliva is mainly a mixture of the secretions from the three pairs of major salivary glands, each secreting a characteristic type of saliva. In addition, it contains constituents from the gingival crevicular fluid, from many microbial contaminants in the mouth, and from the desquamated cells of the oral epithelium. Even the relative contributions of the different glands to whole saliva are variable, depending upon the types and degree of stimulation and even the time of day. Salivary composition may also change in the presence of a systemic disease [1,13].

Studies of salivary composition may be based on studying whole saliva or ductal saliva obtained from different salivary glands. The development of whole saliva proteome analysis, as well as that of saliva obtained from the different glands, may yield important clues to the health of the oral cavity and even the wider pathogenesis of systemic disease [1,8,13,24].

The rich variety of molecules present in the salivary secretions makes saliva an attractive possible source of disease biomarkers. Over the last few years salivary diagnostics methods have been developed. Saliva has many advantages over serum as a medium for clinical diagnosis. Whole saliva is easy to collect and can be stored and transported at low cost. Unlike blood plasma, it does not clot and is therefore easier to handle. The collection techniques for whole saliva are non-invasive, thus reducing patient discomfort and anxiety. On the negative side, whole saliva usually requires centrifugation or filtration to remove precipitated mucins and cellular contaminants. Such centrifugation may also remove other proteins. Some life-threatening diseases such as neoplasms and some cardiovascular diseases are difficult to diagnose without invasive and complicated clinical tests. Current research is trying to develop easier diagnostic tests using saliva [1,7,21].

Salivary proteins and peptides have been studied with traditional biochemical techniques, including liquid chromatography, gel electrophoresis, capillary electrophoresis, nuclear magnetic resonance, mass spectrography, immunoassays (radio-immune assays, immunoradiometric assays, enzyme immuno-assays, enzyme-linked immunosorbent assays) and lectin probe analysis. Most of these analyses have been aimed to investigate specific salivary protein groups, but some efforts have been made to obtain

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complete analyses of salivary proteins and peptides with proteomic techniques [1].

Comprehensive analysis and identification of the salivary proteome may be necessary in order to understand the pathophysiology of the mouth, and, on the other hand, proteins and peptides in saliva can be used as biomarkers in systemic diseases. Currently, the technology of complex identification of peptides and proteins in human saliva becomes more popular [1].

Salivary proteins are examined using a variety of techniques, either single or combined. One technique involves separation of the salivary proteins by two-dimensional electrophoresis and detecting them with a suitable stain. Proteins and their isoforms are usually separated by this technique. Each protein spot can then be excised from the gel and digested with tryptic enzymes [1,13,21].

The proteins, which are separated by the polyacrylamide gel electrophoresis can be more accurately characterized and identified using mass spectrometry. Mass spectrometry has become one of the core technologies in proteomics because of its sensitivity in mass measurement of peptides and proteins with a high degree of accuracy [2].

PURIFICATION OF PROTEINS ISOLATED BY COLUMN CHROMATOGRAPHY

Prior to isolation of the proteins from the solution, their purpose, required degree of purity and activity should be determined. The final product may have three degrees of purity: very high >99%, high 95–99% and moderate <99%. The acceptance of a certain degree of purity determines the type of contaminants that remain in the preparation. Only impurities that do not exhibit biological activity, such as protease, may be accepted [14,19].

For proper isolation of proteins present in biological material, it is important to know the characteristics of the target protein and impurities that must be removed. This regards both separation techniques and the conditions of isolation, so that the loss of proteins caused by inactivation of the protein was minimal. In order to receive the desired protein and biological activity of intact structure, conditions similar to natural occurring in biological materials should be provided. Despite great care, purification procedures may cause a change in environmental conditions, which often has a negative impact on the stability of the isolated proteins. Knowledge of environmental parameters critical for the stability of the protein is important in minimizing the changes while selection of extraction buffer and subsequent purification steps. This applies especially to such parameters as pH, ionic strength, reducing properties, the presence of co-factors, or physical factors such as time, temperature, pressure [14,19,26].

Many compounds have stabilizing effect on protein structure. Reducing agents such as dithiothreitol (DTT) or -mercaptoethanol prevent oxidation of the thiol groups, thereby protecting against protein conformational changes and loss of enzymatic activity. Another important factor affecting the stability of the protein is pH. If it is identical to the protein isoelectric point, the protein solubility decreases and it aggregates easily. Another important parameter that should be taken into consideration is the ionic strength of the environment. Improper buffer salt concentration can lead to aggregation of proteins examined. At an ionic strength in the range from zero to physiological values ??(0.15-0.2 M), some proteins tend to form precipitates due to insufficient electric charge which causes repulsion of protein molecules. When salt concentration is too high, the proteins are salting out [19].

If the protein has temperature stability, all operations can be performed at a lower temperature [19].

The important matter is information about sensitivity of the proteins to proteolytic enzymes, resulting in removal of protease or addition of inhibitors. The knowledge of the properties having direct impact on the selection of separation techniques, such as molecular weight (gel filtration), protein surface charge (ion exchange chromatography), affinity (choice of ligand affinity chromatography), hydrophobicity (the choice of the hydrophobic medium) is also important [14].

It is very important to develop analytical methods for monitoring the purification of proteins. This applies both to the protein itself and to methods for identification of impurities. The amount of protein is determined usually by Lowry [14] or Bradford method [2]. The eluate protein can be measured by measuring the absorbance of light of wavelength 280 nm, absorbed by tyrosine, tryptophan and phenylalanine. Other methods may be immunochemical methods such as ELISA and immunoblotting, electrophoretic methods, which are particularly useful in determining the effectiveness of treatment (SDS-PAGE, NATIVE) or HPLC chromatographic methods [15].

In the process of protein purification, it is important to use relatively minimum number of techniques, providing a product with the desired degree of purity and adequate activity. This is due to the risk of losing significant amounts of protein and its activity at various stages of the procedure as well as the ability to extend the term of the study, which is cost-increasing. The key to successful and efficient protein purification is to select the most appropriate techniques, optimize their performance to suit the requirements and combine them in a logical way to maximize yield and minimize the number of steps required [14].

Very often, protein fractionation is carried out using chemicals causing precipitation, such as ammonium sulfate, polyethylene glycol, acetone [14].

Ammonium sulfate is the most commonly used, and the advantage is the ability to maintain the native structure of the protein. This technique uses the phenomenon of decrease in the solubility of most proteins in the presence of high salt concentrations. At a sufficiently high concentration of salt the protein precipitates. The size of hydrophobic regions of the proteins directly affects the application of the necessary salt concentration. Proteins with larger hydrophobic areas salt out faster than those of smaller hydrophobic areas, and therefore the procedure can be applied to fractionate a mixture of proteins. Salting-out is also used in the subsequent purification steps to concentrate a diluted protein solution, because the protein after precipitation can be re-dissolved in a smaller volume of the buffer [14].

Chromatographic techniques are used for the separation of macromolecules based on parameters such as size and shape, hydrophobicity, surface electric charge and affinity [12,14,26].

Gel filtration

Gel filtration, also known as size-exclusion chromatography (SEC), allows the separation of proteins based on their size and shape of the particles. In this technique, a small volume of sample (<5% by volume of the column) is applied to the column. Gel filtration medium grains are porous insoluble polymer, such as polyacrylamide or dextran grains (Sephadex). During the run, small molecules such as ions diffuse into the gel and move more slowly in the column, while larger or elongated particles flow from the column first. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation. Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37°C or in the cold room according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer [9].

Ion exchange chromatography

Ion exchange chromatography (IEX or IEC) separates biomolecules according to differences in their surface charge. IEX chromatography takes advantage of the fact that the relationship between surface charge and pH is unique for a specific protein. In an IEX separation, reversible interactions between charged molecules and oppositely charged IEX media are controlled in order to favor binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium. However, at a pH above its isoelectric point, a protein will bind to a positively charged medium or anion exchanger and, at a pH below its pI, a protein will behind to a negatively charged medium or cation exchanger.

Most frequently, proteins are eluted by increasing the ionic strength (salt concentration) of the buffer or, occasionally, by changing the pH. As ionic strength increases, the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the ionic strength that is needed for elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form [27].

For separating the proteins endowed with a negative charge positively charged column containing diethylaminoethyl groups (DEAE), such as DEAE-cellulose or DEAE-Sephadex may be used. This is the type of anion exchange chromatography. However, for separation of proteins having positive charge negatively charged carboxymethyl groups (CM) such as CM-cellulose or CM-Sephadex medium shall be used. This is the type of cation exchange chromatography [27].

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a separation technique that uses the properties of hydrophobicity to separate proteins from one another. In this type of chromatography, hydrophobic groups such as phenyl, octyl, or butyl, are attached to the stationary column. Proteins that pass through the column that have hydrophobic amino acid side chains on their surfaces are able to interact with and bind to the hydrophobic groups on the column [14,19].

HIC separations are often designed using the opposite conditions of those used in ion exchange chromatography. In this separation, a buffer with a high ionic strength, usually ammonium sulfate, is initially applied to the column. The salt in the buffer reduces the solvation of sample solutes thus as solvation decreases, hydrophobic regions that become exposed are adsorbed by the medium [14,19].

Affinity chromatography

Affinity chromatography (AC) separates molecules based on the reversible interaction between target protein

and the specific ligand attached to a chromatography matrix. Affinity chromatography is one of the most diverse and powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. It is based on highly specific biological interactions between two molecules, such as interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. These interactions, which are typically reversible, are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase. Successful affinity purification requires a certain degree of knowledge and understanding of the nature of interactions between the target molecule and the ligand to help determine the selection of an appropriate affinity ligand and purification procedure [17,23,25].

One of the most effective methods for purifying proteins is immunoaffinity chromatography, using immobilized antibody. Immunoaffinity chromatography (IAC) combines the use of LC with the specific binding of antibodies or related agents. The resulting method can be used in assays for a particular target or for purification and concentration of analytes prior to further examination by another technique [5,6,16].

The high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including, for example, common operations such as the purification of monoclonal antibodies or fusion proteins [5].

The basis for IAC relies on the selective binding of antibodies. This binding is a result of a large variety of noncovalent interactions that can occur between an antibody and an antigen [16]. The two main types of antibodies that are used in IAC are polyclonal antibodies and monoclonal antibodies. Antibodies can be immobilized onto supports by using a variety of techniques that range from covalent attachment to adsorption-based methods.

Applications of IAC include its use in purification, immunodepletion, direct sample analysis, chromatographic immunoassays and combined analysis methods [16].

FURTHER EXAMINATION

Further examination of separated salivary proteins and peptides can be carried out using ionization methods such as electrospray mass spectrometry (ESI) and matrix desorption / ionization technique (Matrix Assisted Laser Desorption Ionisation - MALDI). ESI-MS is the only mass spectrometry technique that allows relatively easy analysis of substances in both aqueous and organic solutions due to the ability of sample ionization at atmospheric pressure [10,13].

MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. It is similar in character to electrospray ionization in that both techniques are relatively soft ways of obtaining large ions in the gas phase, though MALDI produces many fewer multiply charged ions [10,13].

It has been proven that the isotopic labeling of the mixtures is a useful technique for the analysis of the relative expression levels of proteins in complex mixtures of proteins, such as serum, saliva, urine or cell extracts. There are several methods based on the modification factors in the analysis of isotope tracer protein, allowing the determination of the relative or absolute concentration of the complex in a mixture [3].

Progress in mass spectrometry, liquid chromatography, analysis and bioinformatics software allowed analysis of complex peptide mixtures, the possibility of detection of proteins differing by more than eight orders of magnitude [20]. One of the present methods for the characterization of the proteome of saliva is the isotopic labeling liquid chromatography coupled with mass spectrometry. (IL-LC-MS/MS) [17]. The main methods are based on mass spectrometry using isotopic labeling of complex protein mixtures, such as tissue extracts, blood, urine, or saliva in order to identify the various proteins [17]. The analysis may be performed using a mass spectrometer equipped with a capillary chromatography HPLC. HPLC is coupled with a mass spectrometer by ESI head for maximum sensitivity [17]. An advantage of mass spectrometry in conjunction with the LC is the increased sensitivity and chromatography dependant separation of peptides. Thus, even in complex mixtures of proteins, the data obtained from the MS / MS can be used to sequence and identify peptides with a high degree of confidence [17].

Saliva samples are usually collected from non-smokers, free of confounding conditions: periodontal disease, autoimmune disease, a prior history of diseases of the oral mucosa, or current use of potentially confounding medications. Donors should refrain from eating or drinking for at least 1 h prior to donation. After a water rinse, donors allow saliva to collect in their mouths before gently expectorating into a sterile tube of necessary volume [4,22]. In some protocols, stimulated saliva is taken. Usually, a standardized bolus (1 gram) of paraffin or a gum base is used to stimulate saliva secretion [22].

Clarified saliva can be prepared from fresh or thawed whole saliva samples by centrifuging at 3000×g at 4°C for 15 min, followed by 16,100×g at 4°C for 1 min. The supernatant should then be mixed in a 10:1 ratio with denaturing buffer consisting of 4% SDS, 100 mM dithiothreitol and 100mmol/l Tris, pH 7.4. The samples

the should be boiled for 5 min, cooled to room temperature, then added to a centrifugal filter. Two hundred microliters of buffered urea (8 mol/l urea with 100 mmol/l tris pH 8.5) should be added to the sample, and this mixture should be centrifuged at 14,000×g at room temperature for 40min. An additional 200 ěl of buffered urea should be added and the sample should be centrifuged at 14,000×g at room temperature for 40min. The filters should be discarded and the collected peptides should be alkylated, by addition of iodoacetamide in buffered urea to 50mmol/l, in the dark for 20min. MCX cleanup should be performed by diluting the samples to 3 ml with 2% formic acid and H2O to pH ?3. The MCX columns should be equilibrated with 2 ml of 1:1 methanol: water followed by addition of the entire sample, washing with 3 ml of 0.1% formic acid, 2 ml of methanol, and elution with 1 ml of 95% methanol, 5% ammonium hydroxide. The eluted peptides should be dried in a speed-vac, redissolved in water, and quantified by amodified BCA assay, using trypsin-digested saliva as a standard [4].

CONCLUSION

The determination of salivary biomarkers as a means of monitoring general health and for the early diagnosis of disease is of increasing interest in clinical research. Saliva can reflect the physiologic state of the body, including emotional, endocrinal, nutritional and metabolic variations and acts as a source for the monitoring of oral and also systemic health.

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