Preparation and Characterisation of Monoclonal Antibodies against Highly Abundant Proteins in Human Parotid Saliva

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Objective: *To prepare and characterise monoclonal antibodies (mAbs) against highly abundant proteins in human parotid saliva for the depletion of these highly abundant proteins in the future proteomic studies of saliva.*

Methods: *Proteins in human parotid saliva were concentrated by using ultrafiltration and analysed by SDS-PAGE. The protein band between 50–65 kDa was cut, ground and used to immunise BALB/c mice. The mAbs against highly abundant proteins in human parotid saliva were prepared through hybridoma technology and characterised by ELISA and Wetstern blot.*

Results: *Eleven hybridoma cell lines secreting mAbs against highly abundant proteins in human parotid saliva were established, and Western blot assay showed that these antibodies were specific for the highly abundant proteins in human parotid saliva. mAbs against salivary amylase, the most abundant protein in human parotid saliva, were characterised by ELISA and Western blot.*

Conclusions: *mAbs against human highly abundant proteins in parotid saliva were successfully prepared and characterised. The present study provides an approach for using these mAbs for the depletion of highly abundant proteins in human parotid saliva in order to better enrich and visualise lower abundant proteins for the studies of disease-related biomarkers in human saliva proteome.*

Key words: *highly abundant proteins, monoclonal antibody, parotid saliva, proteome*

Human saliva contains a large array of proteins and peptides (salivary proteome), many of which can be very informative for the detection of human diseases. It is increasingly of special interest to identify "salivary biomarkers" as a means of monitoring general health and for early diagnosis of disease, such as oral diseases or systematic diseases that affect the function of salivary glands, e.g. HIV, Sjögren's syndrome, alcoholic cirrhosis, cystic fibrosis, diabetes mellitus, diseases of the adrenal cortex, cardiovascular diseases, and dental caries[1,2]. This interest is based on several advantages of saliva over other body fluids, such as straightforward and non-invasive sample collection, minimal collection-associated risks, sufficient quantities for analysis, the lower costs of storage and shipping[2,3]. Analysis of human saliva proteome has become one of the most promising approaches to discovery of biomarkers for human diseases[4]. The salivary proteins can be studied from multiple variables at the same time, which can help to discover disease-related biomarkers in saliva. Proteomic biomarkers, when combined, are expected to enhance

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the sensitivity and specificity of human disease detection so that patients could be diagnosed and cured in earlier stages of disease[5].

2-DE-MS is a method combining two-dimensional electrophoresis (2-DE) and mass-spectrometry (MS), which is most commonly used in the proteomic study of saliva[2,3,6-9]. However, in the study of saliva proteomics, 2-DE-MS has reached a bottleneck because of the predominance of several highly abundant proteins in human saliva, including salivary amylases, albumins and immunoglobulins[6]. To obtain better 2-DE separation and visualisation, it will be necessary to remove such highly abundant salivary proteins and enrich those in low abundance[6,10].

The technique of monoclonal antibody (mAb) has been widely used in clinical diagnosis and treatment, as well as in research[11-13]. To separate special antigens from complex antigens can be useful not only for the identification of unknown antigens, but also for better analysis, by the depletion of known antigens. Techniques for the removal of highly abundant proteins using mAb-based immunoaffinity resin or columns have been well studied in blood proteomics[10,14], but there has been little research within salivary proteomics.

The purpose of the present study was to prepare and characterise monoclonal antibodies (mAbs) against highly abundant proteins in human parotid saliva for the depletion of these highly abundant proteins in future proteomic studies of saliva.

Materials and Methods

Cell lines

SP2/0 myeloma cells were provided by department of antibody engineering (Beijing Proteome Research Center).

Experimental animals

BALB/c mice (18-20 g, female, 6-8 weeks old) and New Zealand rabbits (female, 2.5 kg) were obtained from department of antibody engineering (Beijing Proteome Research Center).

Sample preparation

Parotid saliva was obtained from a healthy non-smoking male subject in the morning after overnight fasting, using a Lashley cup as described previously[15]. Saliva was collected simultaneously from the right and left sides for 10 min, after stimulation with drops of 2% citric acid onto the middle of the anterior third of the dorsum of the tongue. In order to minimise the degradation of the proteins, protease inhibitor cocktail (Roche, 10 µl/ml whole saliva) was added immediately after sample collection. The sample was kept on ice during the process. Approximately 8 ml of clear whole saliva was obtained from the subject after centrifuging at 1,300 *g* for 5 min. A further centrifugation at 14,000 *g* at 4 ºC for 15 min was performed to remove debris. The sample was then concentrated and desalted by ultrafiltration through a Centriplus centrifugal filter (nominal molecular mass limit, 3,000; Millipore, Bedford, MA, USA). Protein concentration was determined by using a micro-BCA protein assay (Pierce Biotechnology, Rockville, IL, USA). The sample was divided into 500 µl aliquots and stored at -80 ºC.

Antigen preparation

The concentrated parotid saliva was denatured by heating at 100 ºC for 10 min in 10 x gel-loading buffer. The samples were loaded on 1-mm thick, 10% acrylamide gel and separated by electrophoresis at room temperature (RT) until the bromphenol blue reached the anodic border of the gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 using low molecular mass stained markers as standards. There was a wide protein band between 50 and 65 kDa, which was viewed as the highly abundant proteins in saliva. This band was cut out, ground and stored at -20 ºC.

Immunisation

The prepared antigens were used to immunise female BALB/c mice (n = 3) and female New Zealand rabbits (n = 2) by several subcutaneous and intraperitoneal injections. The first immunisation dosage was 0.2 mg per mouse and 1 mg per rabbit. Three weeks later, another immunisation was made with the same dosage subcutaneously and intraperitoneally. The immune response to the antigen was assessed by measuring serum antibody titre using enzyme-linked immunosorbent assay (ELISA) and the immunised effect was verified using Western blot. When a sufficient antibody titre was reached in serum, a booster injection (200 µg concentrated parotid saliva) was given to the mouse with the highest serum antibody titre, intraperitoneally and intravenously (via the tail veins) at 4 days before fusion after 2 weeks following the previous immunisation.

Preparation of polyclonal antibodies against highly abundant proteins in human parotid saliva

The New Zealand rabbits with verified immunisation were killed by aspirating blood from their hearts. Pooled sera were placed for 2 hours at RT, and centrifuged at 2,000 rpm for 20 min. The supernatants contained the polyclonal antibodies against human highly abundant proteins in parotid saliva.

Establishment of hybridoma cell lines secreting mAbs against highly abundant proteins in human parotid saliva

Cells from the spleen of the immunised mouse with the highest serum antibody titre were fused with SP2/0 myeloma cells line in a ratio of 5:1 using 50% (w/v) polyethylene glycol (PEG) (Sigma, St Louis, MI, USA), as previously described by Kohler and Milstein[16]. The fused cells were cultured in HAT selection medium (containing hypoxanthine, aminopterin, and thymidine). After 2 weeks, HT (hypoxanthine thymidine) medium replaced HAT medium, and this was left to culture for a further 10 days. HT medium was then replaced with RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum. Aliquots of hybridoma supernatants were tested by indirect ELISA to detect specific antibodies. Selected clones were subcloned by limiting dilution. The hybridoma cell lines secreting mAbs against highly abundant proteins in human parotid saliva were allowed to establish until there were sufficient and stable levels of secreting antibody.

Ascitic fluid preparation

Liquid paraffin was injected into the male BALB/c mice intraperitoneally, and 10 days later hybridoma cells were injected into the abdomen cavities of these mice (0.5 ml per mouse, approximately 5 x 10^5-10^6 cells). Ascitic fluid was aspirated when the mice abdomens visibly swelled, and centrifuged at 2000 rpm for 20 min. The antibody titres were determined using indirect ELISA. Isotype determination of mAbs was performed with ELISA.

Characterisation of polyclonal antibodies by Western blot

The concentrated parotid saliva was mixed with 2 x loading buffer in both the presence (reduced) and absence (non-reduced) of 1.5% ß-mercaptoethanol. The mixtures were boiled at 100 ºC for 10 min to prepare reduced and non-reduced samples. SDS-PAGE was performed on 10% acrylamide gel. The proteins separated by electrophoresis were transferred onto a PVDF membrane using mini trans-blot apparatus (Bio-Rad) at 18V for 30 min. Non-specific binding on the membrane was blocked with 5% skimmed milk in PBS-T (0.1% Tween 20 in PBS, pH 7.3) for more than 1 hour at RT. The membrane was cut into strips and incubated individually with: a) immunised rabbit serum (dilution 1:1,000) as first antibody; b) un-immunised rabbit serum (dilution 1:1,000) as negative control; and c) TBS-T as blank. The reactions proceeded overnight at 4 ºC. After four 15-min washes in TBS-T, goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP) (Sigma) as secondary antibody was applied at 1:10,000 for 1 hour at RT. The strips were washed three times for 15 min each in TBS-T followed by a 15-min wash in TBS, and were put together as one membrane, as per the original. The bindings of polyclonal antibodies to antigens were detected using the enhanced chemiluminescence (ECL®) substrate (Amersham).

Characterisation of mAbs by Western blot

Reduced (presence of 1.5% ß-mercaptoethanol) and non-reduced (absence of 1.5% ß-mercaptoethanol) forms of the concentrated human parotid saliva and the commercial salivary amylases were loaded onto 0.75-mm thick gels for electrophoresis and then transferred onto PVDF membranes for Western blot. The supernatants of culture media (dilution 1:1) or ascitic fluid (gradient dilution from 1:250 to 1:8,000) of positive hybridoma cell lines (MGD071) and negative hybridoma cell lines (MGA108) to commercial salivary amylases by ELISA were used as first antibodies. Un-immunised mouse serum (dilution 1:1,000) was used as negative control, fusion mouse serum (dilution 1:1,000) as positive control, and sheep anti-mouse IgG-HRP conjugate (dilution 1:7,500) as secondary antibody. Other operations were the same as for the characterisation of polyclonal antibodies.

Results

Analysis of SDS-PAGE of concentrated human parotid saliva by ultrafiltration

The concentrated human parotid saliva was shown as one wide protein band between 50 and 65 kDa, which was interpreted as the position of highly abundant proteins in human parotid saliva (Fig 1).

Determination of the mouse's serum titre and verification of immunisation effect after the second immunisation

The highest mouse serum antibody titre was 1:64,000 after the second immunisation. Western blot showed there was one specific band between 50 and 65 kDa in accordance with the position of the gel cut out (Fig 2).

Development of hybridoma cell lines secreting mAbs against highly abundant proteins in human parotid saliva

Eleven hybridoma cell lines secreting mAbs against highly abundant proteins in human parotid saliva were established. One of the hybridoma cell lines was characterised by ELISA as it secreted mAbs against salivary amylases, the most abundant protein in human parotid

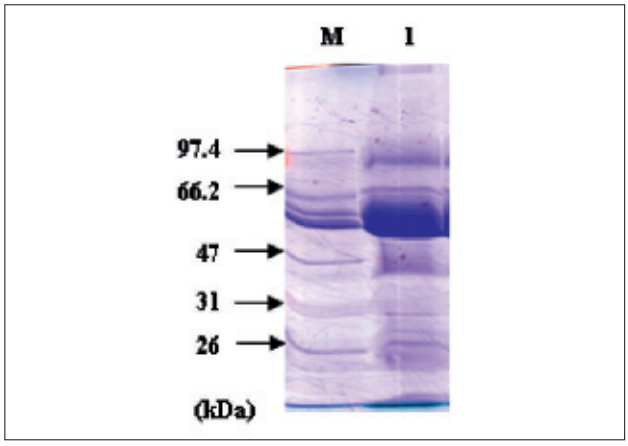

Fig 1 SDS-PAGE of proteins in concentrated parotid saliva. 20 μg proteins were electrophoresed on 10% SDS-PAGE under reducing conditions. Lanes: M, standard; Lane 1, proteins in concentrated parotid saliva.

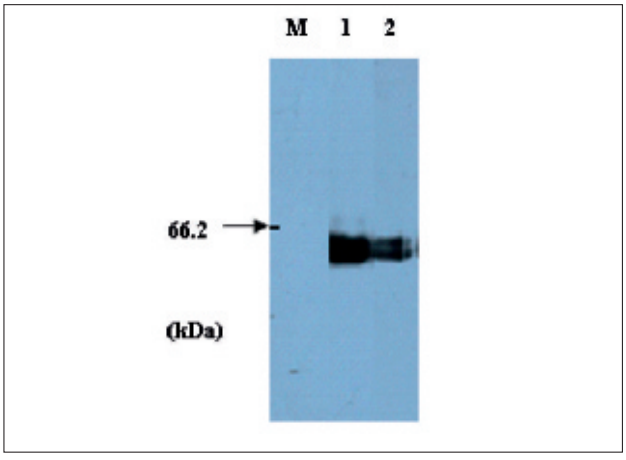

Fig 2 Immunised effect was verified using western blot. Proteins (2 μg) in concentrated parotid saliva were electrophoresed on 10% SDS-PAGE under reducing conditions and transferred onto a PVDF membrane. Membrane strips were individually incubated with immunised rabbit serum and mouse serum. Lanes: M, standard; Lane 1, rabbit serum; Lane 2, mouse serum.

saliva. This hybridoma cell line (MGD071) was IgG1, which provided further clues for the future antibody purification.

Characterisation of polyclonal antibodies by Western blot

Western blot under both the reduced and non-reduced conditions showed that the recognised band by the polyclonal antibody was in accordance with the band in the gel cut out (Fig 3), which indicated that the preparation of polyclonal antibodies against highly abundant proteins in human parotid saliva was successful.

Characterisation of mAbs by Western blot

Western blot under both reduced and non-reduced conditions (Figs 4 to 6) showed that the monoclonal antibodies (either in culture media or in ascitic fluid) positive to salivary amylases by ELISA displayed regional visualisation regardless of whether concentrated human parotid saliva or commercial salivary amylases. The banding patterns were very similar, but the molecular masses varied slightly; commercial salivary amylases showed a relatively lower molecular mass (Figs 4 to 6). It was also shown by Western blot that monoclonal antibodies (either in culture media or in ascitic fluid) negative to salivary amylases but positive to parotid saliva by ELISA were the same reaction as that in ELISA. Non-reduced forms of both the concentrated human parotid saliva and the commercial salivary amylases were more sensitive than the reduced forms.

Discussion

One strategy for enriching lower abundance proteins in saliva is to use mAb-based immunoaffinity resin or columns to remove highly abundant proteins for better enrichment and visualisation of lower abundant proteins in human saliva, according to the specific reactions of antigen-antibody[17]. Many studies indicate that immunoaffinity based methods are effective in selective removal of highly abundant proteins and are gaining considerable importance for the identification of lower abundance proteins in biological fluids[17]. The present study focuses mainly on such biological fluids as serum, plasma or urine, and applicable commercial removal kits have been reported in related studies[18,19]. Commercial mAbs against salivary amylases are rare and the removal effects are not clear. Therefore, preparation and characterisation of mAbs against human highly abundant proteins in parotid saliva against salivary amylases have significance in future proteomic studies of saliva.

In the present study, parotid saliva was used because of its several advantages compared with whole saliva, submandibular and sublingual saliva, such as easier sample collection, less interference from bacterium-related proteins for its amicrobial attribute, easier analysis for the results[8] because of little interference by external environments, and similar distribution of the highly abundant proteins in SDS-PAGE. The classic method of preparing mAbs is to use known proteins or peptides as

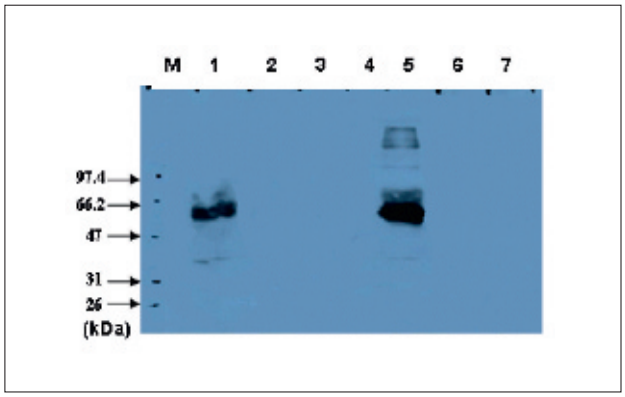

Fig 3 Characterisation of polyclonal antibodies by Western blot. Lanes: M, standard; Lanes 1–3, reduced parotid saliva; Lanes 5–7, non-reduced parotid saliva. Lane 1 and 5, immunised rabbit serum as first antibody; Lane 2 and 6, un-immunised rabbit serum as negative control; Lane 3 and 7, TBS-T as blank. Goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP) (dilution 1:10,000) as secondary antibody.

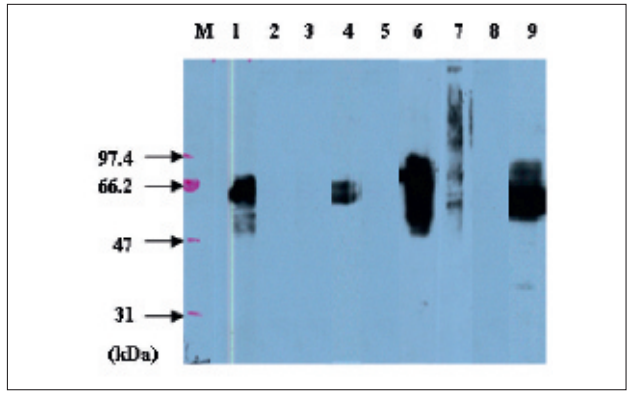

Fig 4 Characterisation of monoclonal antibodies by Western blot. Lanes: M, standard; Lanes 1–4, 0.4 μg of reduced parotid saliva; Lanes 6–9, 0.4 μg of non-reduced parotid saliva. Lanes 1 and 6, the supernatants of culture media (dilution 1:1) of positive hybridoma cell lines (MGD071) to commercial salivary amylases using ELISA were used as first antibody; Lanes 2 and 7, the supernatants of culture media (dilution 1:1) of hybridoma cell lines (MGA108) negative to commercial salivary amylases using ELISA were used as first antibody; Lanes 3 and 8, un-immunised mouse serum (dilution 1:1,000) was used as negative control; Lanes 4 and 9, fusion mouse serum (dilution 1:1,000) was used as positive control.

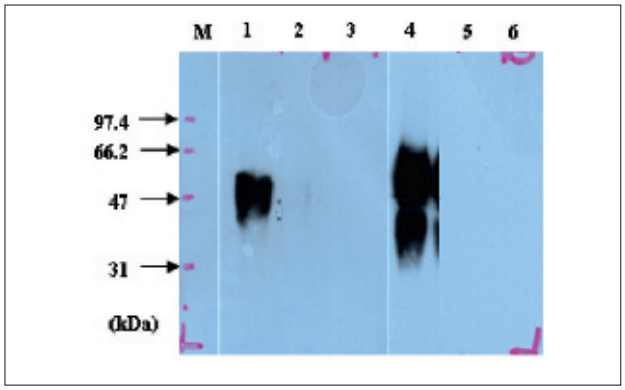

Fig 5 Characterisation of monoclonal antibodies by Western blot. Lanes: M, standard; Lanes 1–3, 0.8 μg of reduced commercial salivary amylases; Lanes 4–6, 0.8 μg of non-reduced commercial salivary amylases; Lanes 1 and 4, the supernatants of culture media (dilution 1:1) of positive hybridoma cell lines (MGD071) to commercial salivary amylases using ELISA were used as first antibody; Lanes 2 and 5, the supernatants of culture media (dilution 1:1) of hybridoma cell lines (MGA108) negative to commercial salivary amylases using ELISA were used as first antibody; Lanes 3 and 6, un-immunised mouse serum (dilution 1:1,000) was used as negative control.

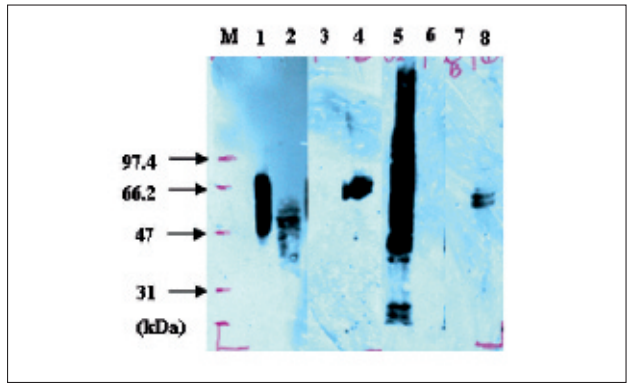

Fig 6 Characterisation of monoclonal antibodies by Western blot. The reduced samples of the concentrated human parotid saliva and commercial salivary amylases were loaded for electrophoresis and then transferred onto a PVDF membrane for Western blot. Positive (MGD071) and negative (MGA108) ascitic fluid (dilution 1:500) to commercial salivary amylases using ELISA was used as first antibodies. Lanes: M, standard; Lane 1, 0.1 μg of concentrated human parotid saliva was loaded; Lanes 3, 4, 5, 7 and 8, 1μg of concentrated human parotid saliva was loaded; Lanes 2 and 6, 0.4 μg of commercial salivary amylases were loaded. Lanes 1 and 2, positive ascitic fluid as first antibodies; Lanes 5 and 6, negative ascitic fluid as first antibodies; Lanes 3 and 7, un-immunised mouse serum (dilution 1:1,000) as negative control; Lanes 4 and 8, fusion mouse serum (dilution 1:1,000) as positive control.

immunogen. However, in the present study, the highly abundant proteins in parotid saliva between 50 and 65 kDa are not clear except for salivary amylases[6]. So using gel bands as immunogen to immunise animals provides a better solution for preparation of mAbs against unknown complex antigens. We separated proteins in parotid saliva by SDS-PAGE and cut gel slabs as immunogen. The existence of specific band between 50 and 65 kDa in accordance with the gel cut out in SDS-PAGE after secondary immunisation showed that our immunisation effect was reliable and effective.

There are 5 or 6 isozymes including glycosylation and deamidisation of salivary amylases shown by SDS-PAGE[20]. These isozymes have close, but different, molecular masses and similar epitopes. In the analysis of Western blot, the mAb showed a regional visualisation instead of a specific band either to concentrated parotid saliva or to commercial salivary amylases. The preparation of mAbs using gel bands as immunogen instead of commercial antigen can better show the true state of the sample. In addition, the regional visualisation also indicates our acquired mAbs against salivary amylases have an immune response to many varied isozymes of salivary amylases, which provides could be useful in the further removal of salivary amylases in parotid saliva.

Summary

We have successfully prepared and characterised mAbs against highly abundant proteins in parotid saliva. mAbs against salivary amylase, the most abundant protein in human parotid saliva, were characterised by ELISA and Western blot. The present studies may provide a useful tool to remove highly abundant proteins in human parotid saliva using mAbs in order to better enrich and visualise lower abundant proteins for the studies of disease-related biomarkers in human saliva proteome.

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