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Trichloroacetic Acid/Acetone Precipitation Method to Optimize Canine Synovial Fluid for One and Two-Dimensional Gel Electrophoresis Studies

(Kaedah Pemendakan Asid Trikloroasetik/Aseton untuk Mengoptimumkan Cecair Sinovium Kanin dalam Kajian Elektroforesis Gel Satu dan Dua Dimensi)

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ABSTRACT

The challenge associated with the use of synovial fluid for osteoarthritic proteome studies is the optimization step, which involves the depletion of high abundant proteins from the samples. The objective of this study was to develop a cost efficient and effective method to remove albumin from canine synovial fluid for proteome studies. Pooled synovial fluid samples were obtained from clinically healthy dogs (n=5), with no radiographic features of osteoarthritis. The acetone precipitation method and 10% w/v of trichloroacetic acid/acetone were chosen to deplete the albumin from canine synovial fluid and the outcome from the different methods were compared using one dimensional and two-dimensional gel electrophoresis studies. The results showed that the 10% w/v TCA/acetone precipitation method removed highly abundant proteins from synovial fluid for gel electrophoresis studies compared to the acetone precipitation method. The 10% w/v TCA/acetone precipitation method provides an effective method to remove albumin from the synovial fluid using gel electrophoresis, especially two-dimensional gel electrophoresis. The accessibility and cost of TCA and acetone make this method of precipitation a simple and cost-effective technique in preparing a canine synovial fluid for two-dimensional gel electrophoresis analysis.

Keywords: Albumin; biomarkers; gel electrophoresis; osteoarthritis; synovial fluid

ABSTRAK

Satu cabaran dalam kajian proteomik berkaitan osteoartritis dengan menggunakan cecair sinovium adalah langkah pengoptimuman yang melibatkan pengurangan protein berkelimpahan tinggi daripada sampel. Objektif penyelidikan ini adalah untuk mengkaji kaedah yang lebih berkesan dan jimat bagi mengeluarkan albumin daripada cecair sinovium kanin untuk kajian proteomik. Sampel cecair sinovium yang dikumpulkan telah diperoleh daripada anjing yang dikenal pasti sihat secara klinikal (n=5), serta tidak mempunyai sebarang ciri-ciri osteoartritis berdasarkan keputusan radiografi. Kaedah mendakan aseton dan 10% w/v asid trikloroasetik/aseton telah dipilih untuk mengeluarkan albumin daripada sampel cecair sinovium kanin dan hasil daripada kaedah berbeza ini telah dibandingkan dengan menggunakan kajian elektroforesis gel satu dimensi dan dua dimensi. Hasil kajian menunjukkan kaedah 10% w/v TCA/aseton berjaya mengeluarkan protein berkelimpahan tinggi daripada elektroforesis gel berbanding kaedah mendakan aseton. Kaedah 10% w/v TCA/aseton telah berjaya menghasilkan satu kaedah yang berkesan untuk mengeluarkan albumin daripada cecair sinovium, terutamanya untuk kajian elektroforesis gel dua dimensi. Kebolehcapaian dan kos TCA dan aseton membuatkan kaedah mendakan ini lebih ringkas dan jimat dalam penyediaan cecair sinovium kanin untuk analisis elektroforesis gel dua dimensi.

Kata kunci: Albumin; cecair sinovium; elektroforesis gel; osteoartritis; penanda biologi

INTRODUCTION

Osteoarthritis (OA) is an age-related musculoskeletal disease and is commonly characterized by the degeneration of articular cartilage, subchondral bone sclerosis, synovitis, and osteophytosis (van Spil & Szilagyi 2019). This condition is always associated with a high

economic burden because of disability and treatment expenses. In recent years, extensive researches have been performed in human medicine on the diagnostic methods for osteoarthritis (OA), such as delayed gadolinium enhanced MRI (Tiderius et al. 2020; van Tiel et al. 2016) and contrast enhanced computed tomography (Stewart et al. 2019; Wang et al. 2016). However, all these detection methods have certain limitations as the diagnostic modalities are not always available. Hence, the validation for another non-invasive and clinically applicable detection method for OA is of crucial importance (Iolascon et al. 2017).

Proteome studies have been valuable in the discovery of biomarkers used in diagnosing a wide range of diseases, including OA (Mahendran et al. 2017; Peffers et al. 2019). These potential disease biomarkers were discovered from synovial fluid (SF) contained within the synovial membrane. The likelihood of identifying proteins related to the disease itself through using SF would be higher due to the proximity with the affected joint. The challenge associated with the use of SF for proteome studies is the optimization step, which involves the depletion of high abundant proteins from the samples. The synovial fluid contains a lot of proteins, which originates from the synovial membrane, cartilage and serum (Bennike et al. 2014; Cretu et al. 2013; Miller et al. 2020). The high abundant proteins, such as albumin and immunoglobulin mask the signals of lower abundance proteins, which could be crucial biomarkers for joint diseases (Ruiz-Romero & Blanco 2010). Therefore, the depletion of high abundance protein is considered a critical sample preparation step for proteomic analyses and profiling strategies (Warder et al. 2009).

Various methods have been proposed for protein depletion to enhance the low signal proteins. In recent years, novel methods using non-expensive chemical reagents, such as acetone, trichloroacetic acid (TCA), ethanol, and acetonitrile to deplete high abundance proteins, have garnered interest among researchers (Kay et al. 2008; Warder et al. 2009). In 2005, a study described a successful removal of albumin from serum using the modified method of protein precipitation, which involved the use of TCA and acetone (Chen et al. 2005). This modified method removed albumin more efficiently in serum samples compared to the acetone precipitation method and commercial depletion kits. However, this precipitation method has yet to be applied to canine SF. Since SF has a similar composition to serum (Fam et al. 2007), we hypothesized that the TCA/acetone precipitation method will deplete albumin effectively as well. This study aimed to optimize the sample preparation of canine SF using the TCA/acetone method for gel electrophoresis studies. This method will be compared with the acetone precipitation method, which is one of the most widely used methods to optimize body fluid samples.

Materials and Methods

Synovial fluid samples

This study was approved by the Institutional Animal Care and Use Committee (IACUC, UPM/IACUC/AUP-T001/2016). Synovial fluid samples were collected intra-articularly from five cadaver dogs with no clinical evidence of OA. A radiograph of the knee joints was performed before the dogs were euthanized for reasons not related to this study. A pooled sample of SF was obtained and stored at -80 °C until further analysis.

Acetone precipitation

In total, 100 µL of SF was precipitated with cold 100% acetone for 60 min at -20 °C. It was then centrifuged at 14000 rpm for 20 min at 4 °C. The supernatant was decanted and washed with cold 100% acetone three times without disturbing the protein pellet. The micro centrifuge tube was then inverted over a C-fold tissue and air dried for 30 s to remove any traces of acetone.

10% w/v TCA/acetone precipitation

100 µL of SF was precipitated with cold 10% w/v TCA with acetone for 60 min at -20 °C and was then centrifuged at 14000 rpm for 20 min at 4 °C. The supernatant was then decanted carefully without disturbing the protein pellet at the bottom of the micro centrifuge tube. The protein pellets were then washed with cold 100% acetone and were centrifuged again at 14000 rpm for 20 min. The supernatant was decanted again and was then washed three more times to remove leftover traces of TCA. The micro centrifuge tube was then inverted over a C-fold tissue and was air dried for one minute to remove any traces of acetone.

Protein quantification

Protein pellets obtained were subsequently suspended in phosphate buffer saline (PBS) and were solubilized with rehydration buffer, consisting of 7M Urea, 2M Thiourea, 4% CHAPS and 0.002% Bromophenol blue. Protein concentration was determined using the 2D Quant Kit (GE Healthcare, Uppsala, Sweden). A total of 5 µL of SF sample was prepared per tube for the assay. Five hundred microliters of precipitant were added to each

tube, including the standard tubes and were vortexed briefly before incubating at room temperature for 2-3 min. The tubes were then centrifuged at 14000 rpm for 5 min to sediment the protein. The supernatant was decanted carefully, making sure not to disturb the protein pellet at the bottom of the micro centrifuge tube. One hundred microliters of the copper solution and 400 µL of ultrapure water were added to each tube and were briefly vortexed to dissolve the precipitated protein. Approximately 1 mL of the working reagent was added to each tube and was mixed immediately by inversion. The tubes were then incubated at room temperature for 20 min. The absorbance was then measured at 480 nm using the Cary®50 UV-Vis spectrophotometer (Varian Inc., CA, USA).

ONE DIMENSIONAL GEL ELECTROPHORESIS

The protein pellets from each of the methods were resuspended with PBS. Then, approximately 30 µg of SF protein was added to 10 µL of 2X Laemli sample buffer (65.8 mM Tris-HCI, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) (Bio-Rad Laboratories, CA, USA). The mixture was then heated at 95 °C for 4 min. The samples were loaded into casted polyacrylamide gels with a Kaleidoscope™ Standards protein ladder (10-250kDa) (Bio-Rad Laboratories, CA, USA). Protein separation was achieved by applying an electrical field for one hour at 120V, 250mA using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, CA, USA). The gel was transferred onto a tray and washed with ultrapure water. The gels were then stained with Bio-Safe™ Coomassie (Bio-Rad Laboratories, CA, USA) and were later de-stained with ultrapure water for 30 min. The gels were analysed using the Gel Doc™ XR+Gel Documentation system (Bio-Rad Laboratories, CA, USA).

TWO-DIMENSIONAL GEL ELECTROPHORESIS

The second dimension was performed with 125 µg of SF protein that was rehydrated overnight on 7 cm Immobiline™ DryStrip gels (IPG) (GE Healthcare, Uppsala, Sweden) with a linear pH range of 3-10. The IPG strip was then focused at a voltage gradient from 5000 to 6500 Vh with a limiting current of 50 mA/strip. After focusing, the proteins were reduced by equilibrating the IPG strips with Dithiothreitol (DTT) equilibrium solution (50 mM Tris-HCl pH 8.8, 6M Urea, 30% glycerol, 2% SDS, 0.002% Bromophenol blue) for 15 min, and then was alkylated with Iodoacetamide (IAA) in equilibration solution for another 15 min. Electrophoresis in the second dimension was carried out on 12% gradient slab polyacrylamide gels at 15 mA/gels until the dye front reached the bottom of the gel using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, CA, USA). The gels were stained with 50 mL of Bio-Safe™ Coomassie (Bio-Rad Laboratories, CA, USA) and the images were acquired using the GS-800™ Calibrated Densitometer (Bio-Rad Laboratories, CA, USA).

RESULTS

In total, a pooled sample of 1 mL of synovial fluid was collected from five cadaver dogs. None of the dogs showed radiographic features of OA. Prior to running the gel electrophoresis, protein quantification was done using a 2D Quant Kit. Acetone precipitated synovial fluid showed a protein concentration of 3.7 mg/mL where TCA/acetone precipitated synovial fluid has a protein concentration of 3.2 mg/mL.

For one-dimensional gel electrophoresis, vertical smearing was observed in Lane 1 (acetone precipitated synovial fluid) compared to Lane 2 (10% w/v TCA/acetone precipitated synovial fluid), which was cleaner and clearer (Figure 1). In one-dimensional gel electrophoresis (1DGE) image, the number of visible bands in the acetone precipitation and TCA/acetone precipitation were 12 and 9 bands. The bands between 10 kDa and 20 kDa were more visible in Lane 1 compared to Lane 2 as can be seen in Figure 1. The two bands between 10 kDa and 25 kDa in size were visible in Lane 1 and were no longer visible in Lane 2. This indicates that some of the lower molecular weight proteins were also depleted in the TCA/acetone precipitation method. Based on previous studies by Chen et al. (2005) and Yamagiwa et al. (2003), albumin has a molecular weight of 66 kDa, which was observed between the 50 kDa and 75 kDa bands based on the protein ladder. In the acetone precipitation method, there was a large protein band with a relative density of 6.5 times larger compared to the TCA/acetone precipitation method.

For the two-dimensional gel electrophoresis (2DGE) results, the gel with TCA/acetone precipitation method (Figure 2(B)) showed minimal horizontal streaking (indicated by the arrows on both Figure 2(A) and 2(B)) with clearer and well-defined protein spots compared to the gel with the acetone precipitation method (Figure 2(A)). The presence of horizontal smearing could be due to large amounts of high abundant proteins. Additionally, a huge protein spot at 66 kDa and p*I* value of approximately 4 to 7 was observed, as shown in Figure 2(A) (indicated by the arrowhead). Based on previous

2DGE studies, this protein is albumin (Chen et al. 2005). The size of this spot is greatly reduced in the gel with the TCA/acetone precipitation method (indicated by the arrowhead in Figure 2(B)). The result from the 2DGE is similar to that in the 1DGE analysis.

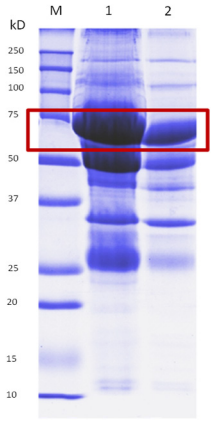

FIGURE 1. One-dimension gel electrophoresis results of (1) acetone precipitated synovial fluid and (2) 10% w/v TCA/acetone precipitated synovial fluid. The band thickness of albumin was greatly reduced in Lane 2 compared to Lane 1. TCA; trichloroacetic acid, w/v; weight/volume

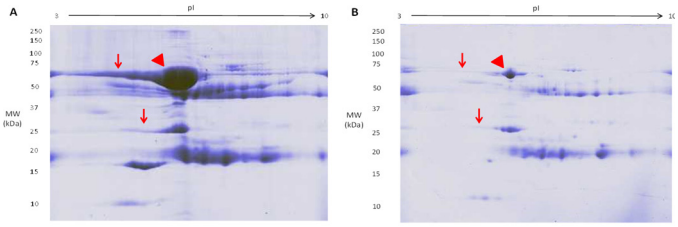

FIGURE 2. Two-dimension gel electrophoresis of canine synovial fluid with (A) acetone precipitation method and (B) 10% w/v TCA/acetone. Albumin spot (arrowhead) was greatly reduced in size after precipitation with 10% w/v TCA/acetone. In (A), horizontal streaking can be seen, which could be due to protein overload or salt content. TCA; trichloroacetic acid, w/v; weight/volume

DISCUSSION

Acetone had been widely used in proteomic studies as a protein precipitation method. However, in this study, acetone was not able to completely remove albumin from SF, which corroborates with the results reported by a previous study (Chen et al. 2011). However, TCA can specifically bind to albumin to form a TCA-albumin complex, which is soluble in organic solvents (i.e. acetone) (Chen et al. 2011), thus, effectively removing albumin from the sample. The removal of albumin in the SF may enhance the presence of other low abundance proteins. However, the depletion of albumin might also remove certain proteins because albumin also acts as a protein carrier (Chen et al. 2011), which can be observed in the TCA/acetone precipitation method shown in Figure 2. Some of the lower abundance proteins smaller than 25 kDa were lost in the TCA/acetone precipitation method. The 2DGE images from the TCA/acetone precipitation method produced gels with clear and defined proteins spots with minimal horizontal streaking. In contrast, the

2DGE images of the acetone precipitation gel produced a large protein spot of albumin, which obscured the other protein spots.

Currently, there are commercially available depletion kits for depleting high abundance proteins. However, these kits either remove only one or two high abundant proteins or remove up to 14 high abundant proteins. These kits provide an easy and convenient pretreatment method of removing high abundant proteins to enhance the presence of lower abundance proteins. Nevertheless, removing large amounts of high abundance protein may lead to a loss of certain proteins (Chen et al. 2005; Fernandez-Costa et al. 2012; Yamagiwa et al. 2003). A study in optimizing cerebrospinal fluid for proteomic studies reported a loss of certain proteins during the process (Chen et al. 2006). Certain protein spots also showed a decrease in protein intensities from samples depleted using the commercial kit (Chen et al. 2011). Consequently, some researchers had opted not to use any precipitation methods in fear of losing important and useful biomarkers (Bennike et al. 2014). Chen et al. (2005) reported that the TCA/acetone precipitation method showed better performance in depleting albumin compared to the depletion kit. The results from the current optimization study also showed that the TCA/acetone precipitation was a better alternative to the acetone method in removing albumin from SF. It provides an effective method to remove albumin from SF for gel electrophoresis, especially for 2DGE. In conclusion, the 10% w/v TCA/acetone precipitation method provides an effective method to remove albumin from SF for gel electrophoresis, especially for 2DGE. The accessibility and cost of TCA and acetone make this method of precipitation a simple and cost-effective technique in preparing canine SF for 2DGE analysis.

Conclusion

The 10% w/v TCA/acetone precipitation method is an easy and cost-effective technique for two-dimensional gel electrophoresis analysis in the preparation of canine synovial fluid.

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