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Isolation and Analysis of Proteoglycans and Glycosaminoglycans from Archaeological Bones and Teeth

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[Abstract] We have developed methods for isolating proteoglycans and glycosaminoglycans from archaeological bones and teeth. These methods have been previously published (Coulson-Thomas *et al.*, 2015) and are described here in more detail. In the case of glycosaminoglycans, the method was a previously described method (Nader *et al.*, 1999) which we optimized for archeological samples.
Keywords: Proteoglycans, Glycosaminoglycans, Archaeological bones, Archaeological teeth, Isolation

[Background] Bone tissue consists mainly of a mineral component (hydroxyapatite) and an organic matrix comprised of collagens, non-collagenous proteins and proteoglycans (PGs). As a result of binding tightly to hydroxyapatite, extracellular matrix proteins and PGs are protected from the destructive effects of temperature and chemical agents after death. However, to date only DNA and proteins had been successfully extracted from archaeological skeletons, and we therefore developed methods for isolating PGs and glycosaminoglycan (GAG) chains from archaeological bones and teeth. PGs and GAGs play a major role in bone morphogenesis, homeostasis and degenerative bone disease, and the analysis of these molecules from archaeological skeletons would unveil valuable paleontological information.

Materials and Reagents

1. Face masks, gloves and clean laboratory coats
2. Safety glasses
3. Autoclaved sandpaper (medium sheet - 100)
4. Autoclaved cheesecloth
5. Autoclaved A4 paper
6. Autoclaved pipette tips
   0.1-10 µl (Eppendorf, catalog number: 022491300)
   2-200 µl (Eppendorf, catalog number: 022491334)
   50-1,000 µl (Eppendorf, catalog number: 022491351)
7. DNA grade 15 ml tubes
8. Amicon® Ultra-15 centrifugal filter unit (3K pore size) (EMD Millipore, catalog number: UFC900308)

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

9. Poly-Prep® chromatography column (Bio-Rad Laboratories, catalog number: 7311550)
10. 50 ml tubes
11. Parafilm
12. Guanidine hydrochloride (Sigma-Aldrich, catalog number: 177253 or G4505)
    Note: The product "177253" has been discontinued.
13. Sodium phosphate monobasic (NaH_2PO_4) (Sigma-Aldrich, catalog number: S8282)
14. Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: RES20908-A7)
15. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E9884)
16. Autoclaved MilliQ water
17. cOmplete proteinase inhibitor cocktail EDTA-free (Roche Diagnostics, catalog number: 05056489001)
18. Urea (Sigma-Aldrich, catalog number: U5378)
19. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S3014)
20. Sodium acetate (CH_3COONa) (Sigma-Aldrich, catalog number: S2889)
21. Maxatase (Biocon Laboratories, São Paulo, Brazil)
22. Trichloroacetic acid (Sigma-Aldrich, catalog number: T6399)
23. Methanol (Sigma-Aldrich, catalog number: 34860)
24. Standard chondroitin sulfate (extracted from shark cartilage) (Sigma-Aldrich, catalog number: C4384)
25. Standard dermatan sulfate (Sigma-Aldrich, catalog number: 1171455)
26. Standard heparan sulfate (extracted from bovine kidney) (Sigma-Aldrich, catalog number: H7640)
27. Standard hyaluronic acid (extracted from human umbilical cord) (Sigma-Aldrich, catalog number: H1751)
    Note: This product has been discontinued.
28. Low-m_r agarose (Bio-Rad Laboratories, catalog number: 1620100)
29. Tris(hydroxymethyl)aminomethane (Tris) (Sigma-Aldrich, catalog number: 252859)
30. Tween 20 (Bio-Rad Laboratories, catalog number: 1706531)
31. EDTA III (Sigma-Aldrich, catalog number: EDFS)
32. Sodium azide (Merck, catalog number: 106688)
33. Bovine serum albumin (Sigma-Aldrich, catalog number: A9418)
34. Sodium hypochlorite solution (see Recipes)
35. Maxatase solution (see Recipes)
36. Blocking buffer (see Recipes)

Equipment

1. Pipettes
   0.5-10 µl (Eppendorf, catalog number: 4920000024)

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

2-20 µl (Eppendorf, catalog number: 4920000040)
20-200 µl (Eppendorf, catalog number: 4920000067)
100-1,000 µl (Eppendorf, catalog number: 4920000083)

2. Autoclave
3. Drill (DRAPER TOOLS, catalog number: 79340)
   Note: It is used with sterilized mounted grinding stones (cylindrical and large cone shaped for bones, and tree and large cone shaped for teeth) (immersed in sodium hypochlorite solution for 15 min, dried and autoclaved).
4. Tube rotator
5. SpeedVac concentrator (*e.g.*, Thermo Fisher Scientific, Thermo Scientific™, model: Savant™ SC210 P1 SpeedVac™, catalog number: SC210P1-115)
6. Water bath
7. Fume hood
8. Ice maker
9. Microcentrifuge, capable of reaching up to 16,000 *x g*
10. Centrifuge, capable of reaching up to 2,000 *x g*
11. Vortex mixer
12. Laboratory oven
13. Quick Scan 2000 densitometer (Helena Laboratories, model: QuickScan 2000)
14. Elisa ELX 800 Wallac Victor2 1420 Multilabel Counter (PerkinElmer, model: Elisa ELX 800 Wallac Victor2 1420)
15. Magnetic stirrer

Software

1. Windows-based Quick Scan 2000 software (Helena Laboratories)

Procedure

A. Bone and tooth preparation
   Note: The investigator must wear a face mask, gloves and clean laboratory coat to ensure no contamination of the samples. These must be changed between samples. The benchtop must be cleaned as described below between samples and new (cleaned and sterilized) tools used for each sample. Modern (control) samples should be handled in a different location on a different day to the archaeological samples.
   1. Clean the benchtop with sodium hypochlorite solution (prepared as described in Recipes).
   2. Clean the archaeological bones and teeth using sterilized water and autoclaved cheesecloth to remove dirt.
   3. Allow to air dry (on the benchtop).

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

4. Clean the bone samples (but not teeth) using autoclaved sandpaper.
   Note: Since tooth enamel forms a hard, smooth surface, sandpaper doesn't have much of an effect on it and dirt particles can be removed by simply using the autoclaved cheesecloth.
5. Wipe the bones and teeth with sodium hypochlorite solution using autoclaved cheesecloth, making sure the samples are exposed to sodium hypochlorite for 15 min for sterilization.
6. Allow to air dry (on the benchtop).
7. Place the bone and tooth samples on autoclaved A4 paper.
8. Wearing safety glasses, drill the bone and tooth samples using the draper drill and sterilized mounted grinding stones (previously immersed in sodium hypochlorite solution for 15 min, dried and autoclaved) until the necessary amount of bone or tooth powder has been collected in DNA grade 15 ml tubes (25-100 mg of bone or tooth powder/tube).
9. Store the bone powder and tooth powder at -20 °C until further processing.
   Note: Approximately 25 mg of bone or tooth powder will provide one PG or GAG sample later on for agarose gel electrophoresis.

B. Proteoglycan extraction

Note: The investigator must wear a face mask, gloves and clean laboratory coat to ensure no contamination of the samples. Modern (control) samples should be handled in a different location on a different day to the archaeological samples.

1. Suspend 25-100 mg of the bone or tooth powder prepared above in 4.5 ml of a solution of 4 M guanidine-HCl, 20 mM NaH_2PO_4, 30 mM Na_2HPO_4, 300 mM EDTA, pH 7.4, containing cOmplete proteinase inhibitor cocktail.
   Note: The solution to suspend the bone or tooth powder is prepared with MilliQ water and contains one tablet of cOmplete proteinase inhibitor cocktail added to 50 ml of solution, as per the manufacturer's recommendations.
2. Maintain under constant rotation at 4 °C for 24 h to keep the solution moving and prevent sedimentation of the bone or tooth powder (*e.g.*, on a tube rotator in a cold room).
3. Add 9 ml of a solution of 7 M urea, 0.3 M NaCl, 0.05 M CH_3COONa, pH 6.5, containing cOmplete proteinase inhibitor cocktail.
   Note: The solution is prepared with MilliQ water and one tablet of cOmplete proteinase inhibitor cocktail added to 50 ml of solution, as per the manufacturer's recommendations.
4. Maintain under constant rotation at 4 °C for an additional 24 h to keep the solution moving and prevent sedimentation of the bone or tooth powder (*e.g.*, on a tube rotator in a cold room).
5. Filter the solution through a Poly-Prep® chromatography column to remove fine particles at 4 °C (*e.g.*, in a cold room).
6. Concentrate and desalinize the solution using an Amicon® Ultra-15 centrifugal filter unit, at 4 °C, to approximately 500 µl.

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

7. Add cOmplete proteinase inhibitor cocktail (from stock solution 25x conc., prepared according to the manufacturer's recommendations) and vacuum dry in a centrifugal evaporator (freeze dry).
8. Store the crude protein/proteoglycan extract at -20 °C until further processing.
   Note: 25 mg of bone or tooth powder provides approximately 5 µg of PGs.

C. Glycosaminoglycan extraction

Note: The investigator must wear a face mask, gloves and clean laboratory coat to ensure no contamination of the samples. Modern (control) samples should be handled in a different location on a different day to the archaeological samples.

1. Suspend 25-50 mg of the bone or tooth powder prepared above in 1 ml of maxatase solution (prepared as described in Recipes).
2. Incubate the samples in a water bath at 60 °C for 2 days.
3. Add 0.1 sample volume of trichloroacetic acid (TCA), vortex and keep the samples on ice for 15 min.
   Note: TCA should be added using a glass pipet in a fume hood to avoid inhalation of vapours. When handling TCA avoid skin contact, wear safety glasses to protect eyes and wear gloves, preferably nitrile gloves. Always add TCA to the sample and not the reverse.
4. Centrifuge the samples (15 min, 2,250 *x g*, at 4 °C*)* and collect the supernatant (placing it on ice).
5. Slowly add 2 sample volumes of methanol in a dropwise manner whilst vortexing.
   Note: The complete volume of methanol must be added with constant vortexing in an uninterrupted manner.
6. Allow the samples to sit at -20 °C overnight for GAGs to precipitate.
   Note: It is important the samples are handled with care (transported on ice and with no brisk movements) while transported to and from the freezer.
7. Centrifuge the samples (10 min, 2,250 *x g*, at 4 °C).
8. Discard the supernatant by pipetting and dry the pellet at room temperature (until dry) or in a laboratory oven (50 °C for approximately 1 h).
   Note: Remove the lid from the tubes and cover the opening with Parafilm. Pierce the Parafilm to allow the methanol to evaporate.
9. Store the crude glycosaminoglycan extract at -20 °C until further processing.
   Note: 25 mg of bone or tooth powder provides approximately 5 µg of GAGs.

D. PG and GAG analysis

1. For analysis and quantification of PGs and GAGs (chondroitin sulphate, dermatan sulphate and heparan sulphate) by agarose gel electrophoresis as described by Dietrich and Dietrich (1976), suspend (1 µg/µl) the crude protein/PG extract and GAG extract in MilliQ water with or without

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

cOmplete proteinase inhibitor cocktail (from stock solution 25x conc., prepared according to the manufacturer's recommendations), respectively, and apply 5 µl to the agarose gel.

2. For quantification of hyaluronic acid (HA) as described by Martins *et al.* (2003), suspend 20 µg of the GAG extract in 300 µl of blocking buffer (prepared as described in Recipes) for performing the assay in triplicate.

Data analysis

If the GAGs are analyzed and quantified by agarose gel electrophoresis as described by Dietrich and Dietrich (1976), then the dried, stained agarose gels are scanned and quantified using a Quick Scan 2000 densitometer and software program (Helena Laboratories), following the suppliers instructions. The intensity of the sample GAG bands that appear on the gels is compared to standard bands of chondroitin sulfate, dermatan sulfate and heparan sulfate that result from a mixture of these GAGs of known concentration (all 1 mg/ml) applied to the same gels. The total quantity of GAGs is then calculated for 100 mg of bone or tooth powder, and this is then divided by the total quantity of protein extracted from 100 mg of bone or tooth powder to provide a GAG quantity in µg/mg of protein.

If HA is quantified according to the probe-based sandwich ELISA assay described by Martins *et al.* (2003), then the plate is read using an Elisa ELX 800 Wallac Victor2 1420 Multilabel Counter (PerkinElmer) and the quantity of HA is calculated by comparison to standard HA added to the plate to produce a concentration curve (0, 0.48, 1.95, 7.8, 31.2, 125, 500 and 1,000 ng/ml HA). HA is then calculated per mg of protein to provide a quantity in ng/mg of protein.

Recipes

1. Sodium hypochlorite solution (final concentration of 1.25%)
   Dilute laboratory bleach (sodium hypochlorite) 1:10 in autoclaved MilliQ water
   Use within 30 days
2. Maxatase solution ([4 mg/ml] in 0.05 M Tris-HCl buffer [prepared with MilliQ water], pH 8.0, containing 1 M NaCl)
   For a final volume of 100 ml:
   a. Weigh 400 mg of maxatase and 5.84 mg of NaCl
   b. Add the maxatase and NaCl to 0.05 M Tris-HCl buffer, pH 8.0, and mix using a magnetic stirrer
   c. Pour into two 50 ml tubes and centrifuge for 30 min to 1 h at maximum speed
   d. Transfer the supernatant to 15 ml tubes and discard the white precipitate
   e. Store at -20 °C
3. Blocking buffer (pH 7.75)
   0.05 M Tris-HCl

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2201

0.15 M NaCl
0.05% Tween 20
0.02 mM EDTA III
7.7 mM sodium azide
1% bovine serum albumin

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