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Full Length Article

Analysis of multiple protein detection methods in human osteoporotic bone extracellular matrix: From literature to practice

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ABSTRACT

The punctual analysis of bone Extracellular Matrix (ECM) proteins represents a pivotal point for medical research in bone diseases like osteoporosis. Studies in this field, historically done to appreciate bone biology, were mainly conducted on animal samples and, up to today, only a few studies on protein detection in human bone are present. The challenges in bone ECM protein extraction and quantitation protocols are related to both the separation of proteins from the mineral content (i.e. hydroxyapatite) and the difficulty of avoiding protein denaturation during the extraction processes. The aim of the present work was to define appropriate protocol(s) for bone ECM protein extraction that could be applied to investigate both normal and pathological conditions. We compared and optimised some of the most used protocols present in the literature, modifying the protein precipitation method, the buffer used for resuspension and/or the volume of reagent used. Bradford and BCA assays and Western Blotting were used to evaluate the variations in the total protein recovery and the amount of selected proteins (Type I Collagen, TGF-β, IGF-1, Decorin, Osteopontin, Bone Sialoprotein-2 and Osteocalcin). Collectively, we were capable to draw-up two single-extract protocols with optimal recovery and ideal protein content, that can be used for a detailed analysis of ECM proteins in pathological bone samples. Time-consuming multi-extract procedures, optimised in their precipitation methods, are however crucial for a precise detection of specific proteins, like osteocalcin. As the matter of fact, also the demineralization processes, commonly suggested and performed in several protocols, could hinder an accurate protein detection, thus inherently affecting the study of a pathological bone ECM. This study represents a starting point for the definition of appropriate strategies in the study of bone extracellular matrix proteins involved in the onset and maintenance of bone diseases, as well as a tool for the development of customized scaffolds capable to modulate a proper feedback loop in bone remodelling, altered in case of diseases like osteoporosis.

1. Introduction

Bone is composed of 2 phases: approximately 75% wt. inorganic and 25% wt. organic. The inorganic phase contains water and mineral, whereby the main component is hydroxyapatite (HA) constituted by crystalline nanoplatelets containing Ca^{2+} and PO_4^{3-}. The organic phase comprises cells and molecules of the extracellular matrix (ECM). Type I Collagen is the most abundant protein, constituting about 90% of bone ECM, whereas non-collagenous proteins (NCPs) represent the remaining part [1]. These latter molecules are different in function and chemical feature, and can be grouped in growth factors - like Transforming Growth Factor-beta (TGF-β) and Insulin-like Growth Factor 1 (IGF-1), glycoproteins – such as Osteopontin (OPN) and Bone Sialoprotein 2 (BSP-2), proteoglycans - like Decorin (DCN), and γ-carboxyglutamic acid-containing proteins - such as Osteocalcin (OCN) [2–7].

The ECM proteins exert an important role in normal bone metabolism and their unbalance could result in bone diseases like osteoporosis. Therefore, the accurate analysis of bone ECM protein content could represent a pivotal point for medical research in this field [8,9]. In the past, the studies that were considered central to the understanding of bone biology were mainly conducted on animals [10–12]. Currently,

Abbreviations: HA, hydroxyapatite; ECM, extracellular matrix; NCPs, non-collagenous proteins; Col1a1, Collagen 1 alpha 1 chain; Col1a2, Collagen 1 alpha 2 chain; TGF-β, Transforming Growth Factor beta; IGF-1, Insulin-like Growth Factor 1; OPN, Osteopontin; BSP-2, Bone Sialoprotein 2; DCN, Decorin; OCN, Osteocalcin

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few studies on protein detection in human bone have been published [13,14].

Protein extraction from bone ECM and the consequent analysis are challenging processes, and some issues, such as the separation of proteins from the HA nanocrystals and the ability to prevent their denaturation during the extraction processes, have not been completely solved [15–17]. Another issue that should be addressed is the complete removal of all soft tissues and cells, in order to avoid inaccurate results [18].

The study of proteins from decellularized ECM makes difficult the normalization of Western Blotting results, as the usual normalization process based on the expression of endogenous proteins from the cytoskeleton or cytoplasm (i.e. Actin, Tubulin, or GAPDH) is not appropriate in the decellularized matrix analysis [19].

A variety of reagents with different functions has been employed for protein extraction from the bone tissue. Reagents, such as ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid (HCl), have been used to demineralize bone and recover proteins strictly bonded to HA [20–22]. Solutions containing guanidine hydrochloride (GuHCl) [10,20,21], ammonium phosphate and ammonium bicarbonate [9], glycerol [13,23], or surfactants and detergents, like Triton X-100 and sodium dodecyl sulphate (SDS) [13,23] have also been applied to extract and solubilize proteins from bone ECM.

The aim of this study was to define appropriate protocol(s) for bone ECM protein extraction that correctly analyse the bone protein content and could be applied to investigate a pathological condition such as osteoporosis. We, therefore, compared some of the most used protocols present in the literature in order to obtain 8 different proteins (Col1a1, Col1a2, TGF-β, IGF-1, DCN, OPN, BSP-2, OCN) that are implicated in osteoporosis onset and its management [24]. Changes in the protein precipitation method, the buffer used for resuspension and/or the volume of reagents used were tested to evaluate the impact on the total protein recovery and the amount of the selected proteins. Bradford and BCA assays and Western Blotting were performed to estimate the efficiency of each protocol.

2. Materials and methods

2.1. Sample preparation

Bone samples were obtained from one osteoporotic humeral head [25] discarded during prosthetic replacement of the shoulder prosthesis surgery carried out at Istituto Ortopedico Rizzoli. In accordance with the Local Ethical Committee guidelines and with the 1964 Helsinki declaration, informed consent was obtained. The patient was aware that the tissue used for the study represented a discard from the surgical procedure and voluntarily participated in the study (freedom from coercion or undue influence, real or imagined). The humeral head was mechanically deprived of the majority of the soft tissue, washed in PBS 1×, dried and stored at −80 °C until use. Sample treatments were summarized in Fig. 1. Briefly, the bone was broken using a hammer and chisel and 1 g of the sample was collected for each protocol, except for protocol 5 where 100 mg of bone were used. The same starting weight for each sample was used to reduce variations that could influence the protein quantification. To degrease and remove the remaining soft tissues and cells, each sample was immersed in a saline solution at pH 7.2 (0.05 M NaCl, 0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4) with protease inhibitors, sonicated in ultrasonic bath for 1 min (min) and interposed with 1 min in ice, for 5 cycles overall (modified from [18]).

Samples were then powdered in liquid nitrogen by mortar and pestle and the same weight of powder (i.e. 850 mg) was aliquoted in tubes.

2.2. Protein extraction

The obtained bone powder was suspended in solutions for each extraction protocol and processed as described below. Each protocol was tested thrice. All incubation and centrifugation steps during protein extraction were performed at 4 °C where not otherwise specified. Samples were resuspended in the same volume of the final solution. To determine protein concentration, the Bradford and BCA based Lowry assays were performed [26].

2.2.1. Protocol 1 (P1): G-E extracts protocol

G-E extracts protocol was modified from Termine 1980 [18] (Table 1) (Fig. 2). The bone powder was suspended in a G solution, constituted of 4 M Guanidine Hydrochloride (GuHCl) in 0.05 M Tris pH 7.4 and protease inhibitors (all from Sigma-Aldrich), and incubated for 72 h. The solution was then centrifuged at 800*g* for 20 min and the supernatant collected (G extract), whereas pellet was resuspended in an E solution containing 0.5 M EDTA (Sigma-Aldrich) in 4 M GuHCl and 0.05 M Tris pH 7.4 with protease inhibitors, and incubated for 72 h. This E extract was then centrifuged at 12,000*g* for 40 min and the supernatant was collected. Proteins in the G extract were obtained by Acetone precipitation, while proteins in E extract were harvested by Acetone or Trichloroacetic acid and sonication (TCA/sonication) precipitations, as described below. After precipitation, 7 M Urea (Sigma-Aldrich) or distilled water (H_2Od) were used as buffers for resuspending. From P1 the following final extracts were obtained: 1G, 1E1, 1E2 and 1E3 (Table 1) (Fig. 2).

2.2.2. Protocol 2 (P2): G1-E-G2 extracts protocol

Protocol 2 was applied according to Goldberg 1988 et al. [27], who have modified the protocol from Termine 1980 et al. [18], to obtain three extracts (Table 1) (Fig. 2). The bone powder was incubated with the G solution for 48 h and then centrifuged at 1000*g* per 15 min. The supernatant was saved as G1 extract and the pellet was washed twice with 0.05 M Tris-HCl pH 7.4, before incubation with an E solution (0.5 M EDTA in 0.05 M Tris-HCl pH 7.4 containing protease inhibitors) for 48 h. This solution was then centrifuged, and the supernatant stored as E extract. The E pellet was suspended again in the G solution for further 48 h and, after centrifugation, the supernatant was collected as G2 extract. Proteins in the G1, E, and G2 extracts were obtained with Acetone or TCA/sonication precipitations, as described below, or concentrated by Amicon Ultra-4. The buffer used after precipitation and during concentration was 7 M Urea. From P2 it was possible to obtain the extracts 2G1, 2G2, 2E and 2G3 (Table 1) (Fig. 2).

2.2.3. Protocol 3(P3): HCl-SDS extracts protocol

HCl-SDS protocol was modified from Buckley 2010 and Craig 2002 [22,28] (Table 1) (Fig. 2). The bone powder samples were treated overnight with 5 or 20 ml of 0.6 M HCl at room temperature and then centrifuged at 7200*g* for 20 min. The supernatant was collected and the remaining pellet washed with H_2Od and centrifuged thrice as above mentioned. The final pellet was then resuspended in 2% SDS (Sigma-Aldrich) for 48 h at room temperature. Proteins in HCl and SDS solutions were precipitated with TCA/sonication and EtOH precipitations respectively, as described below. Proteins from TCA/sonication precipitation were resuspended in 7 M Urea, while proteins from EtOH precipitation were resuspended in 2% SDS. From P3, the extracts 5-HCl, 20-HCl, 5-SDS, and 20-SDS were obtained (Table 1) (Fig. 2).

2.2.4. Protocol 4 (P4): NaOH-EDTA protocol

Protocol 4 was modified from Singh 2011 et al. [29] (Table 1) (Fig. 2). We performed this protocol to determine whether the EDTA solution, used in most protocols for collagen recovery, could hamper protein content, thus reducing their correct analysis and quantification. The bone powder was incubated in 0.1 M NaOH overnight and centrifuged at 3200*g* for 20 min and discarding the supernatant containing NCPs. The pellet was washed twice with H2Od and then resuspended in 0.5 M EDTA pH 8 containing protease inhibitors for 72 h. The sample was centrifuged as described above and the supernatant was collected.

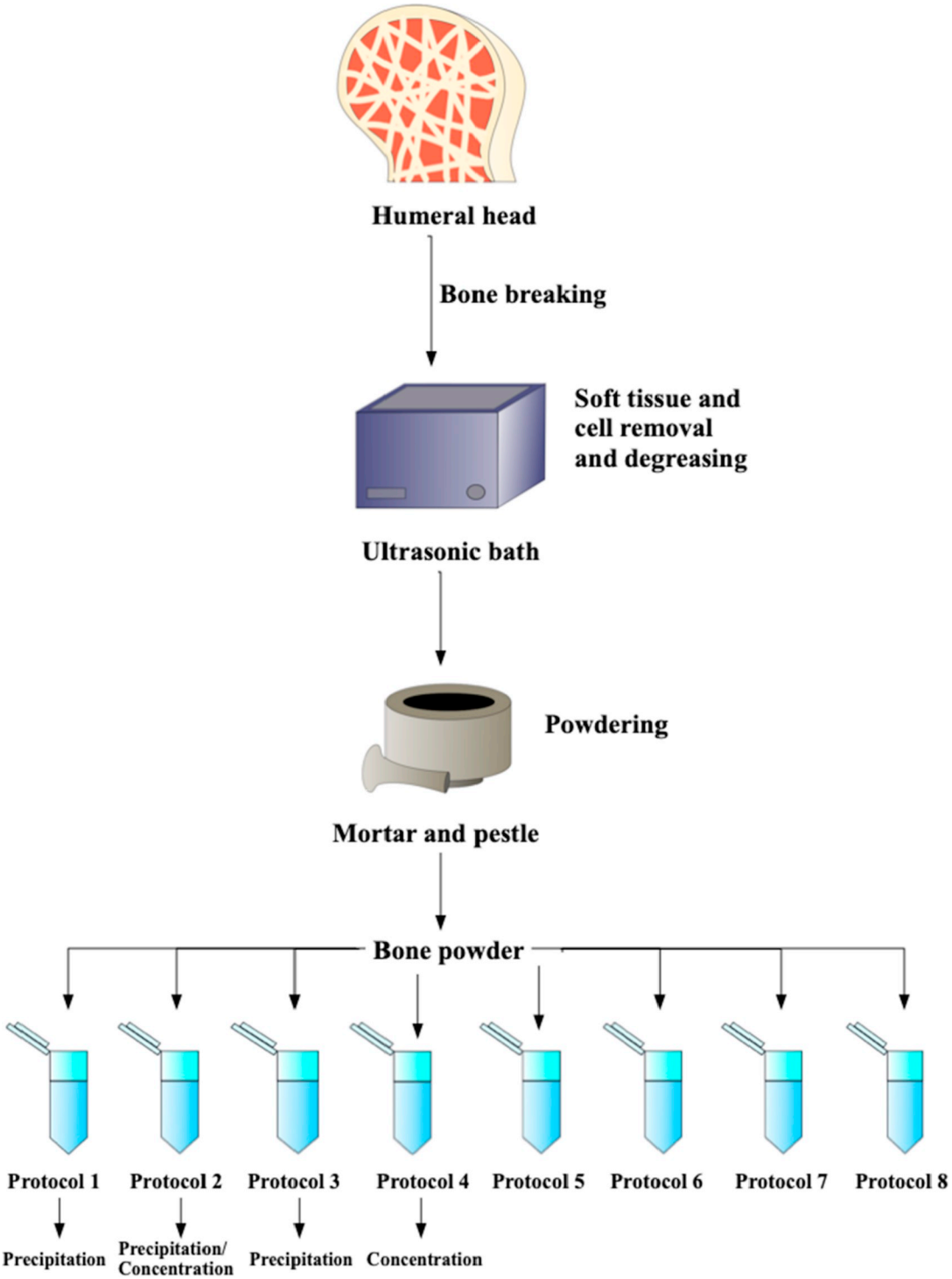

Fig. 1. Representative drawing of stages of the protocols: bone breaking, removal of soft tissues and cells, bone powdering, and processing by different protocols. The first 4 protocols need precipitation and/or concentration methods to obtain protein extracts.

Two consecutive incubations with the EDTA solution were performed. EDTA solution extracts were then concentrated by Amicon Ultra 4 progressively changing the initial buffer with 7 M Urea (Table 1) (Fig. 2). From P4, the extracts 1-EDTA and 2-EDTA were obtained (Table 1) (Fig. 2).

2.2.5. Protocol 5 (P5): TRIzol reagent protocol

TRIzol protocol was conducted according to the manufacturer's instruction (Table 1) (Fig. 3). Briefly, the bone powder (100 mg) was dissolved in 1 ml TRIzol reagent and chloroform (Sigma-Aldrich). After 2–3 min incubation at room temperature, the solution was centrifuged at 12,000*g* for 15 min and the aqueous phase containing RNA discarded. Absolute ethanol was then incorporated, incubated at room temperature and centrifuged at 2000*g* for 20 min, to obtain the protein phase as supernatant. To precipitate proteins, isopropanol was added, and the solution was centrifuged at 12,000*g* for 10 min. The obtained pellets were washed with 0.3 M GuHCl in 95% Ethanol, then centrifuged at 7500*g* for 5 min after incubation and the supernatant was discarded. This step was repeated for 3 times, before incubating with 100% Ethanol for 20 min, centrifuging at 7500*g* for 5 min and

Table 1
Protocols for protein extraction.

Protocols		Starting solutions		Precipitation/concentration method	Final buffer	Final extracts	Days	Ref
N°	Definition	N°	Acronyms					
P1	G-E	2	G	Acetone precipitation	H_2Od	1G	7	Termine 1980
			E	Acetone precipitation	Urea 7 M	1E1		
				Acetone precipitation	H_2Od	1E2		
				TCA/sonication precipitation	Urea 7 M	1E3		
P2	G1-E-G2	3	G1	TCA/sonication precipitation	Urea 7 M	2G1	7	Goldberg 1988 et al
				Acetone precipitation	Urea 7 M	2G2		
			E	Amicon Ultra 4-MWCO1000	Urea 7 M	2E		
			G2	TCA/sonication precipitation	Urea 7 M	2G3		
P3	HCl-SDS	2	5	TCA/sonication precipitation	Urea 7 M	5-HCl	8	Craig 2002; Buckley 2010
				EtOH precipitation	2% SDS	5-SDS		
			20	TCA/sonication precipitation	Urea 7 M	20-HCl		
				EtOH precipitation	2% SDS	20-SDS		
P4	NaOH-EDTA	1	EDTA	Amicon Ultra 4-MWCO1000	Urea 7 M	1-EDTA	8	Singh 2011 et al
				Amicon Ultra 4-MWCO1000	Urea 7 M	2-EDTA		
P5	TRIzol reagent	1	T	N.A.	1% SDS	T	1	N.A.
P6	NET-Triton buffer	1	N	N.A.	N.A.	N	3	Wang 2014
P7	High concentration phosphate buffer	1	HCP	N.A.	N.A.	HCP	3	Cleland et al. 2015
P8	Tris-SDS buffer	1	TS	N.A.	N.A.	TS	3	Lyon 2016

N.A. = not applicable.

discarding the supernatant. In the end, the pellet was resuspended in 200 µl of 1% SDS, the solution was centrifuged at 10000*g* for 10 min to remove insoluble materials and the supernatant stored at −80 °C until use. From P5, T extract was obtained (Table 1) (Fig. 3).

2.2.6. Protocol 6 (P6): NET-Triton buffer protocol

NET-Triton buffer protocol was modified from Wang 2014 [13] (Table 1) (Fig. 3). The bone powder was incorporated in the NET-Triton buffer (0,01 M Tris-HCl 7.4, 1 mM EDTA, 0,1 M NaCl, 1% Triton X-100, 10% glycerol, 0,1% SDS, 0,5% sodium deoxycholate and protease inhibitors) and the solution was homogenized by Ultra-Turrax T8 (IKA-WERKE, Lille, France) for 1 min in ice interposed by 1 min of rest, for 4 cycles overall. The sample was centrifuged at 14,000*g* for 10 min to remove debris and the supernatant was collected at −80 °C until use. From P6, N extract was obtained (Table 1) (Fig. 3).

2.2.7. Protocol 7 (P7): high concentration phosphate buffer protocol

High Concentration Phosphate protocol was modified from Cleland et al. 2015 [9] (Table 1) (Fig. 3). Concisely, the bone powder was dissolved in an HCP solution pH 8 and incubated at 75 °C for 24 h. The HCP solution contained 400 mM ammonium phosphate dibasic, 200 mM ammonium bicarbonate and 4 M GuHCl. The samples were then centrifuged at 14,000*g* for 30 min to precipitate insoluble materials and the supernatant was collected and stored at −80 °C. From P7, the HCP extract was obtained (Table 1) (Fig. 3).

2.2.8. Protocol 8 (P8): Tris-SDS buffer protocol

Tris-SDS buffer protocol was modified from Lyon 2016 [23] (Table 1) (Fig. 3). The lysis buffer contained 1.2 M Tris-acetate pH 6.8, 1% SDS, 0.5% glycerol, 1% 100 mM EDTA and Protease Inhibitors (Sigma-Aldrich), 10 mM β-glycerophosphate, 1 mM sodium orthovanadate and 50 mM dithiothreitol (DTT) were added immediately before use. The solution containing bone powder was homogenized by Ultra-Turrax T8 for 1 min in ice interposed by 1 min of rest, for 4 cycles overall, and frozen at −80 °C overnight. The sample was then centrifuged at 14,000*g* for 10 min to remove mineral matrix debris and the supernatant was collected at −80 °C until use. From P8, TS extract was obtained (Table 1) (Fig. 3).

2.3. Precipitation methods

All passages for TCA/sonication or Acetone precipitation were performed at 4 °C where not otherwise specified [30]. Briefly, for TCA/sonication precipitation protocol, 1 volume of 20% TCA was added to 1 volume of protein solution and incubated at −20 °C for 1 h. The solution was centrifuged at 15,000*g* for 15 min and the supernatant was removed. Ice-cold acetone containing 20 mM DTT was added to the pellet, incubating at −20 °C for 1 h and vortexing every 20 min, and then the solution was centrifuged at 13,000*g* for 15 min. The supernatant was discarded, and the pellet was air-dried before resuspending in the selected buffer and sonicating for 10 s for 6 cycles overall. For Acetone precipitation protocol, 4 volumes of ice-cold acetone containing 20 mM DTT were added to 1 volume of protein solution. The solution was vortexed and incubated at −20 °C for 1 h. The supernatant was discarded after centrifuging at 15,000*g* for 15 min, and the pellet was air-dried before resuspending in the selected buffer.

Ethanol precipitation was performed as previously described by Rajalingam 2009 and all the passages were performed at 4 °C where not otherwise specified [31]. Concisely, 5 volumes of 100% ethanol were added to 1 volume of protein and incubated overnight at −20 °C. The solution was centrifuged at 7000*g* for 20 min, the supernatant was wasted, and the pellet washed with 5 ml of ice-cold acetone and incubated for 20 min at −20 °C. The centrifugation was performed at 7000*g* for 20 min, the supernatant was discarded, and the pellet was resuspended in the selected buffer after air drying.

2.4. Western Blotting

Western Blotting was performed on the extracts obtained from three different reps of the above-mentioned protocols. All protein samples (50 µg of protein for each sample) were prepared using NuPAGE™ LDS Sample Buffer according to manufacturer's instruction, fractionated in NuPAGE™ 4–12% Bis-Tris Protein Gels and electrophoretically transferred to PVDF membranes (Millipore).

Membranes were incubated with 5% milk in Tris-Buffered Saline with 0.1% Tween 20 (TBS-T) to block non-specific sites and then with primary antibodies in TBS-T at 4 °C overnight.

After washes with TBS-T, the membranes were incubated with secondary antibody anti-mouse conjugated with horseradish peroxidase or secondary antibody anti-rabbit conjugated with horseradish peroxidase. Detection of antibody binding was performed with Pierce ECL Western Blotting Substrate and images were acquired with Alliance Mini HD9 (Uvitec, Cambridge, UK). Densitometric analysis was performed with ImageJ software (https://imagej.nih.gov/ij/download.

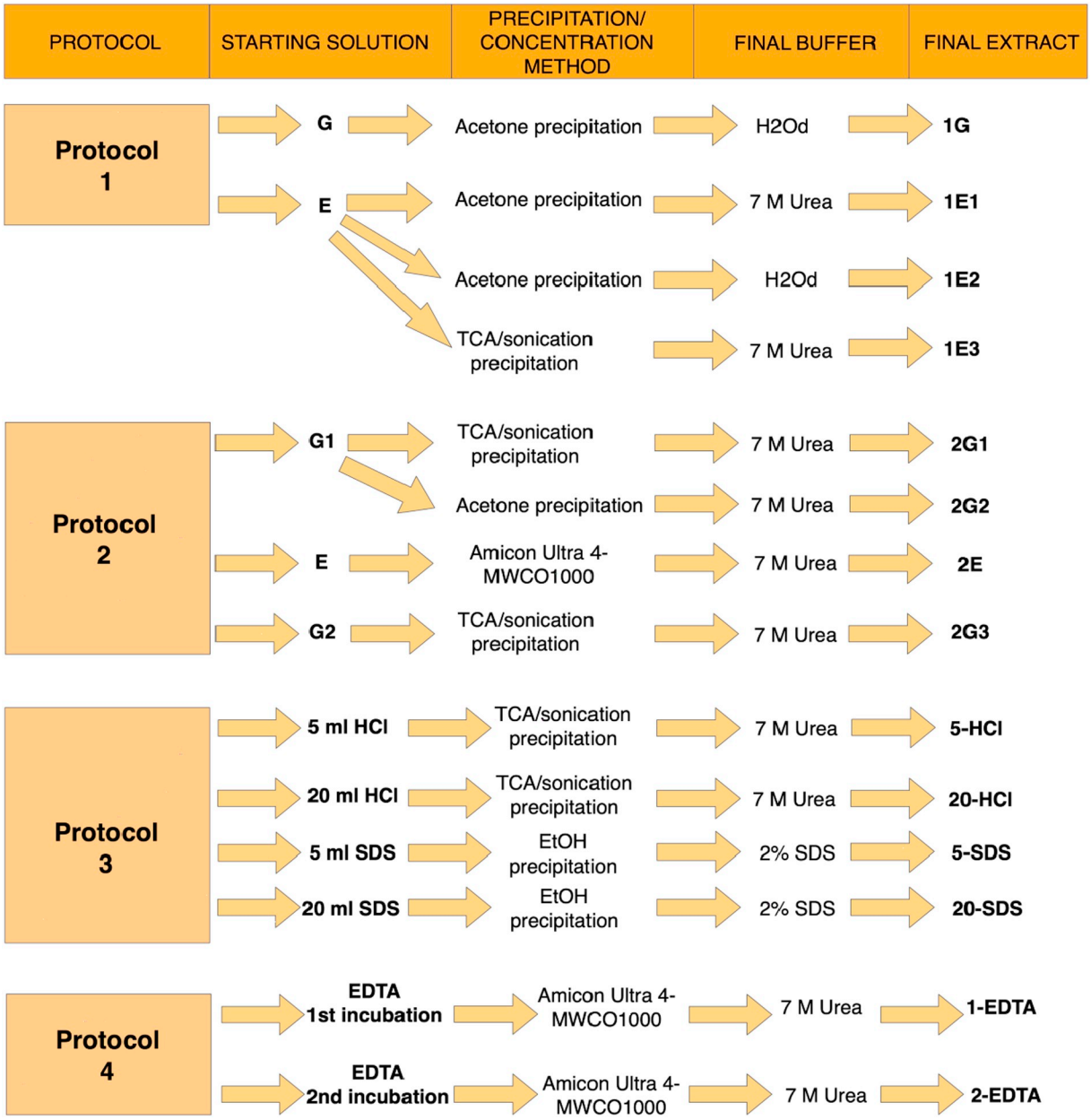

Fig. 2. Schematic drawing of the steps of the multi-extract protocols (P1–P4). For each step of the protocols, starting work solutions, precipitation and/or concentration methods, final buffers employed for resuspension after precipitation or concentration and final extracts obtained are indicated.

html). No membrane was stripped between antibody incubations to avoid protein loss.

2.5. Key resources table

ANTIBODIES				
REAGENT	**ANTIBODY**	**DILUTION**	**CATALOG**	**SOURCE**
Human Col1a1	Mouse monoclonal	1:250	SC-293182	Santa Cruz Biotechnology
Human Col1a2	Rabbit polyclonal	1:1000	14695-1-AP	Proteintech
Human Decorin	Rabbit polyclonal	1:1000	Ab-175404	Abcam
Human Osteocalcin	Mouse monoclonal	1:200	SC-365797	Santa Cruz Biotechnology
Human Osteopontin	Mouse monoclonal	1:200	SC-73631	Santa Cruz Biotechnology
Human Transforming Growth Factor- β	Mouse monoclonal	1:2000	GTX21279	Genetex
Human Insulin-like Growth Factor- 1	Mouse monoclonal	1:250	SC-74116	Santa Cruz Biotechnology
Human Bone Sialoprotein- 2	Mouse monoclonal	1:200	SC-73630	Santa Cruz Biotechnology
Secondary antibody conjugated with horseradish peroxidase	Anti-mouse	1:15000	A190-116P	Bethyl Laboratories
Secondary antibody conjugated with horseradish peroxidase	Anti-rabbit	1:5000	SC-2004	Santa Cruz Biotechnology
CHEMICALS				
Protease inhibitors	N/A	N/A	S8820	Sigma-Aldrich
TRIzol reagent	N/A	N/A	15596018	Invitrogen
Dithiothreitol (DTT)	N/A	N/A	NP0009	Invitrogen
Bradford reagent	N/A	N/A	B6916	Sigma-Aldrich

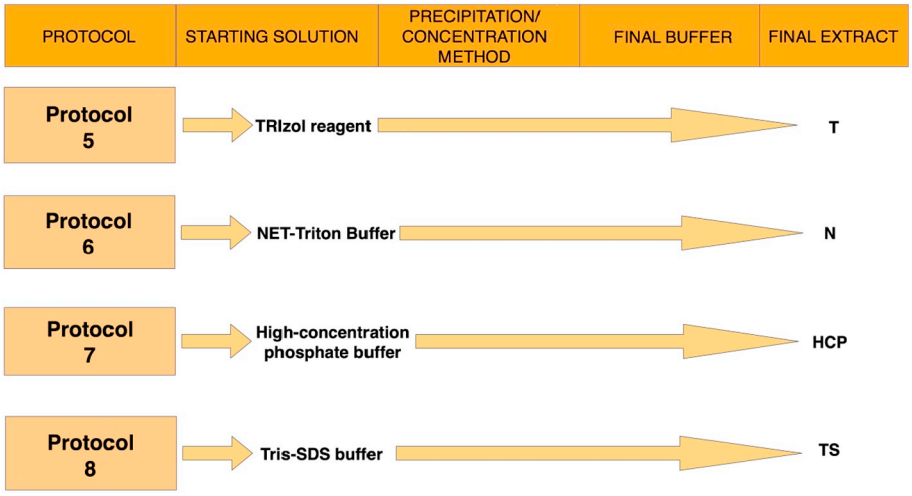

Fig. 3. Schematic drawing of steps of the single-extract protocols (P5–P8). For each step of the protocols, starting work solutions and final extracts obtained are indicated. In this drawing, we have underlined that precipitation or concentration methods and buffer for resuspension were not used.

NuPAGE™ LDS Sample Buffer (4X)	N/A	N/A	NP0007	Invitrogen
NuPAGE™ 4-12% Bis-Tris Protein Gels	N/A	N/A	NP0322BOX	Invitrogen
Pierce ECL Western Blotting Substrate	N/A	N/A	32106	Thermo Scientific
DEVICES				
Amicon Ultra 4	N/A	N/A	MWCO10000	Merck Millipore
Ultra-Turrax T8	N/A	N/A	Z404519	IKA-WERKE

3. Results

3.1. Macroscopic observations

Macroscopically, some solutions showed insoluble residues after protein extraction. The TS and N extracts, obtained from P8 and P6 respectively, exhibited a filamentous residue during the homogenization phase. The 1E2 extract, obtained in P1 from the resuspension in H_2Od after acetone precipitation, contained a copious amount of white residue, whereas the 1E3 extract from P1 gained in the same protocol after the resuspension in 7 M Urea after the TCA/sonication precipitation contained only a little insoluble white deposit.

3.2. Total protein amounts

Table 2 summarized the total protein recovery from each extract, comparing Bradford and BCA assays. The highest protein recovery detected by Bradford assay was in P5 and P2: the protein concentration of the T extract (P5) was 3.450 μg/μl from 100 mg of bone, while the 2G1 extract (P2) contained 15.613 μg/μl from 1 g of bone. A good protein recovery was also detected in the HCP extract from P7 (11.774 μg/μl), the 2G2 extract from P2 (9.625 μg/μl), and the N extract from P6 (8.239 μg/μl).

Further differences were found treating the same extract with different precipitation methods (i.e. 2G1 vs 2G2 extracts) and/or different buffer (i.e. 1E1 vs. 1E2 extracts from P1) or using a different volume of extraction solution (5-HCl vs 20-HCl extracts from P3; 5-SDS vs. 20-SDS extracts from P3). The highest protein content was detected in the T (P5) and the 2G1 (P2) extracts also by BCA assay; overall, this assay showed a lower protein recovery than Bradford one: the protein concentration for the T extract was 1.076 μg/μl from 100 mg of bone and for the 2G1 extract was 1.950 μg/μl. A good protein recovery was observed also in the P extract from P7 (1.540 μg/μl), in the 2G2 extract from P2 (1.460 μg/μl) and in the TS extract from P8 (1.015 μg/μl). We found differences in protein recovery comparing the same extract with different precipitation methods (i.e. 2G1 vs 2G2 extracts from P2) or using a different volume of extraction solution (5-SDS vs 20-SDS from P3). No proteins were detected in 1E3 extract (P1).

Table 2
Total protein recovery.

Protocols		Final extracts	Concentration (μg/μl)	
N°	Definition		Bradford assay	BCA assay
P1	G-E	1G	1.452	0.909
		1E1	1.732	0.280
		1E2	1.024	0.259
		1E3	1.166	–
P2	G1-E-G2	2G1	15.613	1.950
		2G2	9.625	1.460
		2E	1.392	0.335
		2G3	3.951	0.232
P3	HCl-SDS	5-HCl	0.913	0.492
		5-SDS	2.454	0.267
		20-HCl	0.514	0.447
		20-SDS	3.623	0.088
P4	NaOH-EDTA	1-EDTA	2.200	0.933
		2-EDTA	3.539	0.964
P5[a]	TRIzol reagent	T	3.45	1.076
P6	NET-Triton buffer	N	8.239	0.935
P7	High concentration phosphate buffer	HCP	11.774	1.540
P8	Tris-SDS buffer	TS	3.348	1.015

[a] Starting from 100 mg of bone.

3.3. Protein staining analysis

Results of the densitometric analysis are summarized in Fig. 4. Detailed information are available as Supplementary files (Figs. 1S–4S and Table 1S).

Only TS (P8) and T (P5) extracts showed staining for all the examined proteins. The T extract revealed the best staining for TGF-β at 25 and 50 kDa, DCN at 40 kDa, OPN at 55 kDa and BSP-2 at 55 and 80 kDa. The TS extract showed the highest intensity for Col1a1 at 140 kDa, IGF-1 at 7.6 kDa, OPN at 25 and 66 kDa and a similar intensity to the T extract for BSP-2 at 55 kDa. Furthermore, the N extract (P6) exhibited the highest amount of Col1a1 at 90 kDa, TGF-β at 12.5 kDa and BSP-2 at 35 kDa but did not show signals for Col1a2 and IGF-1

	P1				P2				P3				P4		P5	P6	P7	P8
	1G	1E1	1E2	1E3	2G1	2G2	2E	2G3	5-HCl	20-HCl	5-SDS	20-SDS	1-EDTA	2-EDTA	T	N	HCP	TS
Col1a1 140 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ ++	+++ +	-	+++ ++
90 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	+	-	+/-
Col1a2 120 kDa	-	++	++	++	-	-	++	+++	+++ +	-	+	+	+++	+++ ++	+/-	-	+	+++
TGF-beta 50 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ ++	-	-	+++
25 kDa 12 kDa	-	-	-	-	-	-	-	-	++	-	+++ +	+++	-	-	+++ ++	-	-	+++ ++
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-
IGF-1 22 kDa	-	+	-	-	-	-	+	-	-	-	+++ ++	++	-	-	-	-	-	-
7 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	+++
Decorin 120 kDa	-	-	-	-	-	-	-	-	+++ +	+++	-	-	-	-	++	+/-	-	+
40 kDa	-	-	-	-	-	-	-	-	++	+	+++	+	-	-	+++	+/-	-	++
Osteopontin 66 kDa	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	+++ +	-	-	+++ +
44 kDa 25 kDa	-	++	+	+	-	-	+	-	-	-	-	-	-	-	+++ ++	-	-	+/-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ +	+++ +	-	+++ ++
Bone Sialoprotein-2 80 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ ++	-	-	+++ ++
55 kDa 33 kDa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++ +	-	-	+++ +
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+++ +	-	+++
Osteocalcin 6 kDa	+++ ++	+++ +	++	++	+++ ++	+++ +	-	-	+/-	+	+/-	+/-	-	-	+/-	+/-	+/-	+/-

Fig. 4. Schematic drawing to resume the results of densitometric analysis for protein expression of each extract (I: intensity/mm3). −: no staining; +/−: $I < 10^6$; +: $10^6 < I < 2.5 \times 10^6$; ++: $2.5 \times 10^6 < I < 5 \times 10^6$; +++: $5 \times 10^6 < I < 8 \times 10^6$; ++++: $8 \times 10^6 < I < 1.2 \times 10^7$; +++++: $I > 1.3 \times 10^7$.

proteins. Col1a2, TGF-beta, IGF-1, DCN, and OCN were identified in the 5-SDS and 20-SDS extracts (P3). The 5-SDS extract showed also the highest staining for IGF-1 at 22 kDa. The 5-HCl extract (P3) exhibited staining for Col1a2, TGF-beta, OPN, OCN and the highest content of DCN at 120 kDa, whereas the 20-HCl (P3) showed only the presence of DCN and OCN. The 1E1, 1E2 and 1E3 extracts (P1) contained Col1a2 and OPN in similar amounts, but the 1E1 extract contained also IGF-1 and showed staining for OCN 4-fold more than 1E2 and 1E3. The 2E extract permitted the collection of Col1a2, IGF-1, and OPN. The remaining extracts showed staining only for one protein: among these, the 2G1 extract (P2) showed the highest staining for OCN and the 2-EDTA one for Col1a2.

4. Discussion

Protein extraction from bone ECM is a complicated process

influenced by several variables, such as the use of different reagents, the concentration methods and the resuspension buffers, which could hamper the accurate quantitation of many proteins involved in bone biology and pathologies.

In this study, we compared different protocols from published literature to identify the best method for the extraction of bone ECM proteins in a pathological condition (i.e. osteoporosis). Since the imbalance between bone resorption and formation affects the bone ECM protein content [8], the modification of current protocols in terms of total protein recovery and collection of peculiar proteins involved in osteoporosis could represent a valid tool in the study and/or management of this pathology.

The normalization of Western Blotting results was one of the main issues to address because the usual analysis of endogenous proteins cannot be useful in the examination of a decellularized bone ECM.

Therefore, in order to reduce variations that could influence the protein quantification, arrangements like the use of only one humeral head to obtain different samples (each protocol was tested thrice), the same starting weight and final volume of resuspension, an equal protein amount loaded in Western Blotting gels, and no membrane stripping were taken [32,33].

To load the same protein amount we performed Bradford and BCA assays. Matching the same extract, we found a higher protein quantitation with Bradford assay. In bone ECM, Type I Collagen usually represents 90% of the total protein content, and its chains contain arginine and lysine that preferentially bound to Coomassie in the Bradford assay [26,34,35]. The remaining 10% of bone ECM proteins is constituted by 180–200 NCPs [34,35]. Osteocalcin, the most abundant among them, and TGF-β are primarily revealed by BCA assay, whereas IGF-1, DCN, OPN and BSP-2 are preferentially identified by Bradford assay. Considering the protein detection amount and the affinity for the most proteins of interest, Bradford assay was the best for our aim.

Other useful arrangements to reduce influence in protein quantification, save for the suggested above, could be the use of Ponceau or Coomassie normalization for protein loading/transfer measure and the positive control loading for the analysed proteins [36,37].

Protocols P5, P6, P7 and P8, which are single-extract protocols, provide the proteins in a single extract without using precipitation and/or concentration methods and do not require > 3 days. The T extract from P5 showed both the higher total protein recovery and the detection of all proteins of interest, providing the best-tested ones. Also, P6 showed good results in terms of protein content, allowing the collection of most of the selected proteins. On the contrary, although the total protein recovery from P7 was high, the HCP extract contained only Type I Collagen and OCN. Additionally, the TS extract from P8 exhibited similar staining results obtained with P5, but with a 10-fold lower total protein concentration than the latter.

In the T (P5) and TS extracts (P8), that resulted as the best-tested protocols, Type I Collagen was the protein with the highest expression, but OCN staining was very low compared to all the other detected NCPs.

With the multi-step protocols, we obtained several extracts capable to provide different proteins according to their chemical and physical features. With the use of GuHCl buffers for the first step of P1 and P2, we expected to collect proteins from unmineralized tissue and osteoid layer, like proteoglycans and part of Type I Collagen, in the corresponding extracts (1G, 2G1 and 2G2) [18,20]. Nonetheless, the extracts were positive only for OCN which is strictly related to mineralized tissue [38], showing a high content of this protein.

Demineralizing solutions were employed in the first step (as in P3) or after (as in P1, P2 and P4). The extracts including EDTA or HCl contained OCN and other proteins associated with HA, like OPN and Type I Collagen, as expected [18,20,27,38–40]. Interestingly, we also observed the expression of growth factors (i.e. TGF-β and IGF-1) and proteoglycans (i.e. DCN). In the EDTA and HCl extracts derived from demineralizing solutions, we obtained a notable protein amount and variety, as observed also by Schroeter et al. [17]. Furthermore, in P3, the SDS extract, collected after the demineralizing step with HCl solution, exhibited only a low amount of Type I Collagen. Some protocols for protein extraction from bone, especially those employed for collagen collection, use a first demineralizing step on samples before the effective extraction procedures [41,42]. Our data suggest that the initial decalcification step causes loss of proteins, affecting the final protein recovery, and therefore should be avoided.

Lately, from the third step of P2, we recovered the residue Type I Collagen in the 2G3 extract, as expected [27].

Although each multi-step protocol provides two or more solutions for the obtainment of specific extracts, no tested protocol was per se useful to collect all the 8 proteins of interest. Additionally, these multi-step methods require more days and longer period of incubations than the single-extract ones, thus they are not time-efficient. With these protocols (from P1 to P4), just a few extracts exhibited a good protein recovery with a good or high expression of few proteins of interest. Among them, OCN showed a very high expression in the P1 and P2, greater than the single-extract protocols that thus revealed a limitation in the collection of this protein.

The possibility to investigate, within these protocols, different precipitation and/or concentration methods and different buffers for resuspension, could overall ameliorate protein recovery. The TCA/sonication precipitation and the subsequent buffer changing with 7 M Urea provided the best protein recovery and staining in G extracts, while acetone precipitation and 7 M Urea resuspension was the greatest for the recovery and staining in E extracts, even if in the 1E2 and 1E3 extracts (P1) an insoluble white sediment was observed. This deposit that we supposed to be mineral residues, was not evident in the 1E1 extract (P1) obtained with TCA/sonication precipitation.

Also, in the TS (P8) and N (P6) extracts from single step-protocols a filamentous residue, consisting probably of an insoluble fraction of Type I Collagen [18], was observed. Overall, these deposits, which probably contain not solubilized proteins, could affect the proper estimation of protein content in the tested protocol.

Another issue that needs to be addressed in the search of an optimal protocol is the volume of extraction solution. For instance, in P3, the extract from 5 ml HCl was better in quantity and quality than that from 20 ml HCl. Similarly, even if the SDS extracts contained the same proteins, yield and expression were higher in the extract from a pellet that was demineralized by a smaller amount of HCl.

In some extracts (i.e. 2G2 from P2 and HCP from P7), we observed differences between a high total protein recovery, detected by Bradford and BCA assays, and a very low protein expression, observed by Western Blotting. This discrepancy may be attributable to the fact that in these extracts there were other proteins bedside the ones we investigated. Indeed, the investigated proteins relative to osteoporosis are few considering the very high number of NCPs in bone ECM [34]. Otherwise, also the buffer used for extraction and/or for resuspension could cause this discordance. Indeed, solutions containing a high salt amount, as the HCP extract from P7, could be not fully suitable for the SDS-PAGE system in Western Blotting, thus making the protein detection difficult [43]. For this reason, P7 was not optimal for Western Blotting analysis, but its high protein recovery makes it a good protocol to analyse proteins by other assays such as HPLC [9]. On the other hand, the solutions containing SDS, like those used in P3, P5, P6 and P8, were compatible with SDS-PAGE system and showed a wide range of proteins detectable.

5. Conclusions

Collectively, we drew-up two single-extract protocols (P5 and P8) with optimal recovery and ideal protein content for the main proteins involved in osteoporosis and that can be used for a satisfactory analysis of ECM proteins in pathological bone samples. These protocols need, however, to be supported by the multi-extract protocols for a punctual

detection of proteins like OCN (e.g. P1 or P2). We also optimised the precipitation methods in the multi-extract protocols based on the different components of the starting solution employed. We observed that the proposed demineralization processes before the extraction could cause protein loss, thus hampering accurate protein detection.

This study provides useful protocols to investigate the protein content in bone ECM. It serves as a baseline for the definition of appropriate strategies in the study of bone proteins in medical research, to investigate their involvement in the onset and continuation of bone metabolic diseases, like osteoporosis. Furthermore, more information about the protein content of bone ECM could represent a starting point to design appropriate scaffolds. Indeed, the inclusion in biomaterials of one or more crucial proteins can be central to develop in vitro models for physiological or pathological bone conditions and/or for the biofabrication of customized 3D scaffolds capable to properly modulate the feedback loop in bone remodelling altered in osteoporosis.

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CRediT authorship contribution statement

Caterina Licini:Methodology, Formal analysis, Writing - original draft, Writing - review & editing.**Giorgia Montalbano**:Methodology, Formal analysis, Writing - review & editing.**Gabriela Ciapetti**:Writing - review & editing.**Giorgia Cerqueni**:Writing - review & editing.**Chiara Vitale-Brovarone**:Conceptualization, Writing - review & editing.**Monica Mattioli-Belmonte**:Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

None.

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