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ORIGINAL PAPERS

Evaluation of Polypeptide Complexes from Marine Sources with Relevance in Initiation and Propagation Mechanisms of Osteoarticular Dysfunctions

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Abstract. *The aim of the present study was to obtain a protein fraction rich in enzymes with proteolytic activity such as serine proteases, chymotrypsins and cysteine proteases and to investigate preliminary pharmacological effects with therapeutic potential in osteoarticular diseases. The processing of marine raw material by applying established and optimized sequences of operations (selective precipitation followed by fractionation by FPLC) led to well-defined protein fractions rich in proteolytic enzymes. To evaluate the biological effect of the isolated polypeptide fractions, an in vitro model on normal human osteoblast (HOB) and osteoarthritic (HOB-OA) cell lines was carried out in which the modulation of cell proliferation under the action of the polypeptide complexes was followed.*

Key words: osteoarthritis, trypsin proteinase, HOB-OA, proteases.

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1. Introduction

Osteoarthritis is a progressive non-inflammatory condition characterized by gradual erosion of articular cartilage until its disappearance, accompanied by osteophytic bone remodeling, subchondral sclerosis, inflammatory changes of the synovial membrane and joint capsule [1-4]. Recently, a serious international

public health problem is emerging, as the prevalence of osteoarthritic disease has been found to be increasing, representing the leading cause of joint pain and disability for adults over the age of 50 [1-4]. These issues have led to the need to identify and develop new therapies that aim to reduce pain, improve function, decrease disability all converging to improve the quality of life of patients with osteoarthritis [3-6]. The synovial joints present a complex structure with the participation of the following elements: the articular surfaces represented by the articular cartilage (the component with the most important role in the functioning of the joints) between which the synovial fluid, the meniscus and the articular capsule are interposed. [7]. The biomechanical functionality of a joint is negatively affected by the degradation of the articular surface due to biological aging processes that are associated with alterations in the metabolism of the articular cartilage [2-6]. These biochemical processes, some probably genetically determined, are strongly influenced by trauma and a multitude of factors that alter the structural or metabolic integrity of articular cartilage [4,5-8]. The most important characteristic of articular cartilage is its remarkable strength and durability in a mechanically demanding environment, the maintenance of the structural integrity of the surface being due to a fragile balance established between the biochemical processes taking place in its metabolism [1-6]. If this balance is disturbed in the sense of accelerated catabolism there is a degradation of the matrix which decreases the resistance of the tissue to mechanical stress thus leading to degradation of cartilage tissue [1-8]. Although a catabolic response of regeneration of matrix, collagen and proteoglycan components is initiated in reaction to this degeneration, unfortunately the delicate metabolic balance of the tissue is not restored [5-8]. For this reason, mechanical damage to a joint surface will lead over time to the premature development of arthrosis, due to the limited potential of articular cartilage to spontaneously repair chondral lesions, and when repair occurs it is partial with fibrocartilage repair tissue, whose mechanical properties are far inferior to hyaline cartilage [7-9]. In addition, all clinical data showed the presence of an inflammatory component in the development and propagation of osteoarticular lesions [1].

A wide range of treatments are used in the management of this lesions, including both conservative therapy (administration of chondroitin sulphate and glucosamine pharmaceutical preparations, hyaluronic acid, steroidal and non-steroidal anti-inflammatory drugs, as well as modern substances such as matrix metalloproteinase inhibitors and growth factors and cytokine inhibitors) and interventional therapy [1,2,6-9].The current treatment for patients presenting with painful episodes is non-steroidal anti-inflammatory drugs (NSAIDs), which belong to the group of drugs that modify rapid onset symptoms and are widely prescribed as the mainstay therapy for symptom relief, although they do not treat the cause [1, 10,11]. Long-term or repeated use of these drugs is often associated

with an increased risk of gastric and duodenal ulcers [1]. Having a very good tolerance, diclofenac is one of the most widely used anti-inflammatory steroidal drugs [1, 11]. At the same time, new therapies are being sought that combine active ingredients with anti-inflammatory and pain-reducing efficacy, offering a much better risk/benefit ratio [1]. In a large epidemiological study, the rutoside - bromelain - trypsin combination was shown to have a superior benefit/risk ratio to the non-steroidal anti-inflammatory drugs present in treatment guidelines for patients with osteoarticular diseases [12]. This has been attributed to the fact that 1) by binding antiproteinases bromelain decreases bradykinin and tipsin improves fibrinolysis and together decreases proinflammatory cytokines (i.e. TNF-a, IL-1, IFN-c) and 2) rutoside has antioxidative properties inhibiting hyaluronidase in connective tissues, blocking ATPases, phospholipases, cyclo-oxygenases and lipooxygenases [1-12]. Although the mechanism of action of trypsin, chymotrypsin and cysteine proteases at the cellular and molecular level is not fully elucidated, the multitude of anti-edematous effects, the action on antiproteinases and a2-macroglobulin, in the context of osteoarthritis may be a particular interest, the combination of both serine proteases and cysteine proteases could be a suitable choice [1].

Taking into account the need, at least at European level, to diversify protein sources in order to reduce their dependence and environmental footprint, new trends in pharmacological research to maximise natural potential without harming the ecosystem are the valorisation of by-products/ industrial waste from the processing of raw materials from marine sources. The aim of the present study was to obtain a protein fraction rich in enzymes with proteolytic activity such as serine proteases, chymotrypsins and cysteine proteinases and to investigate the preliminary pharmacological effects with therapeutic potential in osteoarticular diseases. The effects of polypeptide fractions isolated, purified and characterized from by-products resulting from the industrial processing of marine fish were investigated on an in vitro model for the evaluation of cell proliferation.

2. Material and Methods

2.1 Preparation and qualitative assessment of polypeptide content

The technological process stage of raw material processing have carried out a well-defined analytical screening was performed to determine the protein concentration and molecular mass range. The raw material from the marine source was subjected to a long series of technological processing operations that involved the exhaustive extraction of protein/ peptides from the biological matrix and their separation from contaminating non-protein molecules, thus isolating the total protein mixtures. The total protein solution was treated with ammonium sulphate (60% and 90%) thus achieving a concentration and a pre-fractionation of the

protein component due to the specific properties of these "salting out" molecules. Depending on the degree of saturation in ammonium sulphate applied to the protein solutions, the two mixtures A (for 60% saturation of ammonium sulphate) and B (for 90% saturation of ammonium sulphate) were obtained and then subjected to the fractionation process by using FPLC (Fast Protein Liquid Chromatography) AKTA model, in the following parameters of the system: Superose 6 Increase 10/300 GL column (Size exclusion); 0.010M PBS (phosphate buffered saline), pH=7.4; separation of the compounds was carried out in isocratic mode at a flow rate of 0.5mL/min; total elution volume: 1.2 CV (column volume), flow 0.5 ml/min, detection at 280nm with fraction collection at 1 ml interval.

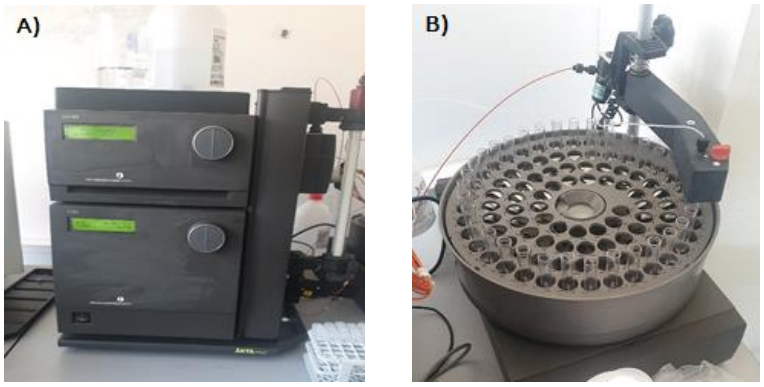

Fig. 1. FPLC AKTA system (UPC-900, P-920, F-900 module) (**A**) with fraction collector (**B**)

Before start the injection of the sample and collect the fractions, the column was washed with 2 x CV with purified water and 1 x CV with PBS. The samples were centrifuged at 10000 rpm for 10 minutes, then filtered by nylon filter with 0.2 µm pore size. The chromatographic profile of mixture of standard (about 100 µg/mL of each standard) acquired during FPLC separation is presented in (Figure.2.), to assess the separation of protein profile by molecular weight and to guide the collection of fractions, from the real sample. The fractions were lyophilization at -55 °C, chamber pressure 0,02 mBar for about 24 hours.

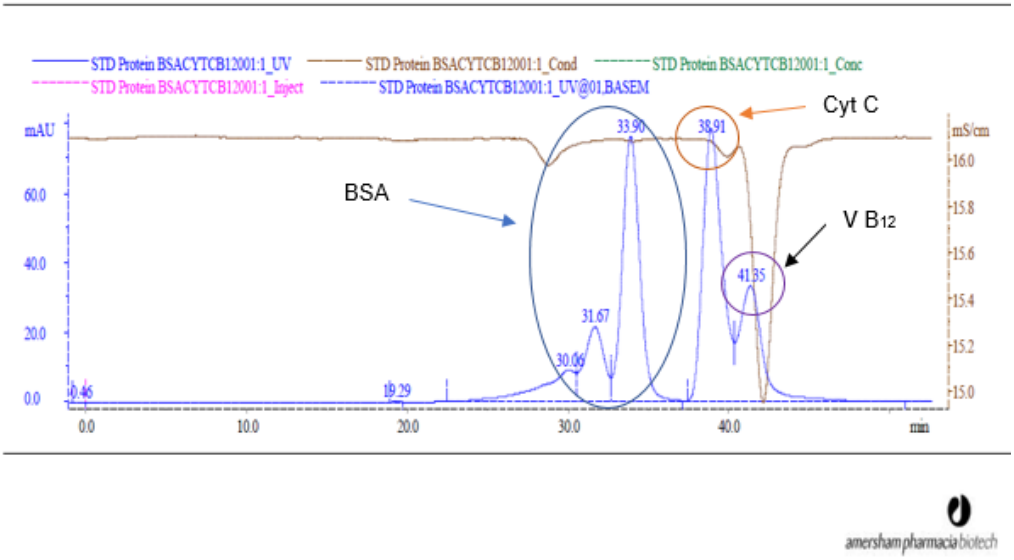

Fig. 2. Chromatographic profile of mixture of standard

Protein assay of lyophilized samples was performed by Bradford method and the molecular mass range was determined by migration of the collected fractions in polyacrylamide gel (method applied: SDS-PAGE Laemmli Buffer System). In order to specifically characterize the protein fractions isolated and characterized with the aim of highlighting their potential biocatalytic properties, an analytical technique for the detection of proteases by zymography using specific substrates (0.1 % gelatin / casein, under nonreducing conditions, run at 60 V in a Tris-glycine buffer) was developed and applied, thus identifying proteins with protease activity of the caseinolytic or gelatinolytic type [13.]. The renatured enzymes exert their proteolytic activity on the copolymerized substrate during 12 hours of incubation at 37 ^{0}C in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.01 M $CaCl_2$ and 1 µM $ZnCl_2$ [14]. Identification of polypeptides with enzymatic activity was performed after staining the gels with 0.25 % Coomassie blue R-250, destaining with methanol: acetic acid: water (40:10:50; v/v) and appear white on a dark blue background. Classification into an enzyme class was made on the use of specific inhibitors. In this way, when the presence of a serine protease in the protein fraction was desired, specific inhibitors for the other protease classes were introduced into the development buffer. The zymograms were scanned with ChemiDoc MP Imaging System from BIO-RAD.

2.2. Evaluation of the biological action of polypeptide complexes

The aim of the research was to evaluate the biological action of polypeptide complexes isolated from industrial fish waste on osteoblastic cell proliferation. The study was carried out on two cell lines of normal (HOB, primary culture) and

osteoarthritic (HOB-OA, at small passages: 2-3) human osteoblasts, cultured for 5 days, in two experimental series: basal conditions (without stimulation) and stimulation with Calcitriol (regulates bone metabolism both indirectly, by controlling calcium and phosphate homeostasis, and directly, at the osteoblast level - the cell that ensures the functionality of the endocrine system of vitamin D) 100mM for 48h. The analysis was carried out by the flow cytometry technique which involves the labeling of nuclei with propidium iodide and the estimation of nuclei accumulation percentages in the three phases of the cell cycle by the analysis of fluorescence histograms.

3. Results and discussion

The development of standardized technologies for the isolation and purification of peptide molecules from marine raw materials offers great potential for the discovery of new active substances that can be introduced into pharmaceutical formulations. Since the purification of proteins in active form is still a major challenge due to the use of techniques that mainly rely on the native properties of proteins, the challenge of this experimental step was to establish the optimal conditions for isolation/purification of polypeptides so that the biological function of the molecules present in the initial extract is not altered. In the development of such a methodology, sample preparation is the fundamental step to perform a valid analysis because the quality of the results obtained depends on the nature of the specimen and the properties of the isolated proteins [15]. Protein mixtures must have a high concentration in the molecules of interest and not contain salts and/or other components that may introduce interferences in the subsequent analysis such as detergents, nucleic acids, lipids. In large-scale processing of biological materials for the concentration and fractionation of target molecules from various contaminants, protein precipitation techniques followed by their resolubilization in a suitable buffer are used [16]. In the framework of the research, several precipitation systems (acetone 80%, TCA 10%, acetone acidified with hydrochloric acid, ammonium sulphate, PEG 600, methanol) were studied in order to achieve these goals, with which, in most cases, the coextracted compounds were successfully removed from the sample with a low degree of recovery of the protein component and with impairment of the biological activities, except for ammonium sulphate. The working conditions in this step were chosen to maintain the proteins in their native state, as far as possible the extract should contain all the proteins of interest in the sample to be analyzed, but as few contaminating molecules as possible. By introducing a pretreatment step with ammonium sulphate, the fractionation process was improved because coextrased compounds of non-protein origin were removed, a concentration and a prefractionation of the protein component was achieved due to the specific properties of these molecules of "salting out", preserving the native form of the

polypeptides and maintaining their biological activity. Figure 3 shows the chromatograms recorded for the separation of the two mixtures resulting from the precipitation of the total protein extract with 60% ammonium sulfate (mixture A) and respectively 90% ammonium sulfate (mixture B) and in Table 1 the protein concentrations for each fraction collected before lyophilization.

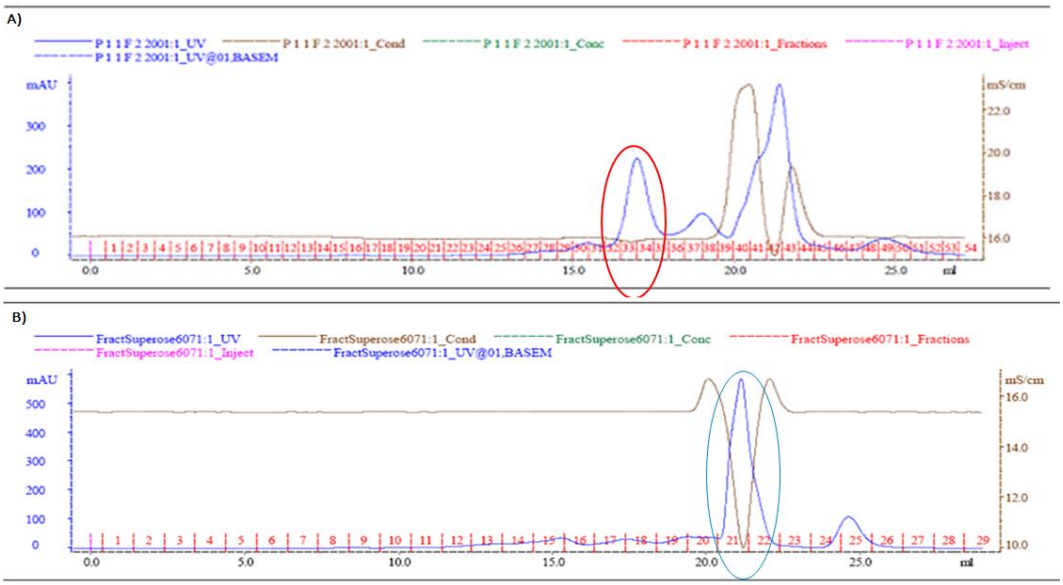

Fig. 3. Chromatographic profile acquired during FPLC separation for: A) A protein mixture and B) B protein mixture

Table 1. Quantitative assessment of protein content

No.	*Sample*	*Protein concentration, mg/ml*
1.	fraction 32 from mixture A	1,048
2.	fraction 33 from mixture A	3,774
3.	fraction 34 from mixture A	2,419
4.	fraction 21+22 from mixture B	6,002

Evaluation of protein fractions collected after FPLC separation in terms of molecular mass distribution and identification of proteolytic activity was performed by electrophoretic techniques such as SDS-PAGE and gelatin zymography (see Fig.4.).

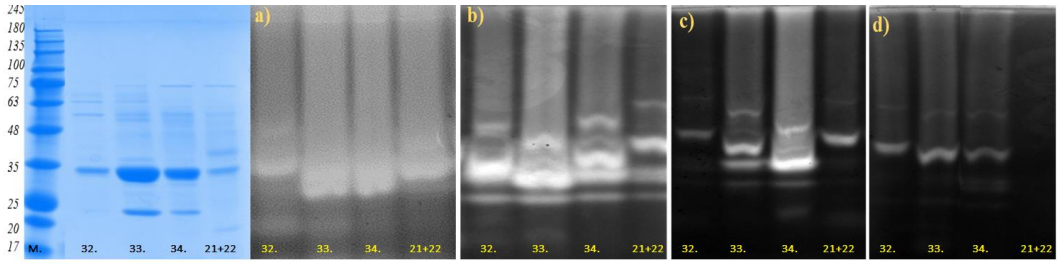

Fig. 4. Protein fingerprinting by SDS-PAGE of fractions collected from separation of A and B mixture. Identification and placement in an enzyme subclass by gelatine-zymography technique of isolated proteases from A and B protein mixture. (a) total proteases; b) serine proteases; c) chymotrypsin-like serine proteases; d) cysteine proteases; (legend: M. molecular market; 32, 33 and 34 - protein fractions collected from protein mixture A below chromatographic peak 1; 21+22 - protein fractions collected from protein mixture B below chromatographic peak 2)

Chromatography fractions showing proteolytic activity were reanalyzed in the presence of specific inhibitors (for serine protease - phenylmethylsulfonyl fluoride, PMSF; for trypsin - tosyl lysine chloromethylketone, TLCK; for chymotrypsin - tosyl phenylalanine chloromethylketone, TPCK; for metalloproteinases - epoxysuccinyl-leucylamido butane, Euc 64 and ethylenediaminetetraacetic acid, EDTA) with the aim of classifying them in an enzyme subclass. The presence in each collected fraction of a given class of proteases was identified by the specific inhibition of protein enzymes of no interest. From the analysis of the zymograms, it is found that the protein fraction 21+22 does not present proteins with enzymatic activity specific to cysteine proteinases.

Based on the experimental results obtained following the studies to confirm the degree of separation of the proteins contained in the isolated fractions, those with biologically active potential were selected and evaluated in vitro on osteoblast cultures (fraction 32 and 33 because they have the highest serine protease and cysteine protease activity). We turned our attention to this cell line because the inflammatory signals in osteoarticular pathology come from the immune system, the immunological signals to the bone being transmitted mainly through osteoblasts to induce the maturation of osteoclasts, triggering secondary osteoporosis [10-14]. Such phenomena occur mainly at the interface between the proliferative synovial environment and bone tissue [10-15]. Osteoblasts not only play an important role in bone formation by synthesizing multiple bone matrix proteins, but also regulate osteoclast maturation through soluble factors and related interactions, resulting in bone resorption [1-5]. The osteoclast maturation process requires stimulation by RANKL expressed on osteoblasts, and the related

interaction is mediated by firm adhesion via ICAM-1 [1-6]. Table 2 briefly shows the results obtained in the cell cycle sequencing tests in which the ability of protein complexes to modulate osteoblastic proliferation in both normal and osteoarthritic culture was monitored. This parameter is extremely important because the reduced proliferation of bone osteoblasts leads to the decrease of the newly formed trabecular bone, highlighted by the reduction of the average thickness of the wall.

Table 2. Osteoblastic proliferation – sequencing of the cell cycle modulated by polypeptide complexes with protease activity

HOB-OA						
No.	***Sample***	***Treatment for 5 days without stimulation***				
		%G0/G1	*%S*	*%G2/M*	*%S+G2/M*	
1.	Cellular control	96.56	0.00	3.45	3.45	100.0
2.	fraction 33 from mixture A	95.88	1.38	2.74	4.12	119.42
3.	fraction 34 from mixture A	95.83	1.28	2.89	4.17	120.87
4.	Vitamin C 0,004%	95.59	1.59	2.82	4.41	127.83
No.	***Sample***	***Treatment for 5 days with stimulation (calcitriol 100mM, 48h)***				
		%G0/G1	*%S*	*%G2/M*	*%S+G2/M*	
1.	Cellular control	96.23	0.91	2.86	3.77	100.0
2.	fraction 33 from mixture A	97.11	0.69	2.2	2.890	76.66
3.	fraction 34 from mixture A	97.22	0.38	2.4	2.78	73.74
4.	Vitamin C 0,004%	96.76	0.55	2.78	3.25	86.21
HOB						
No.	***Sample***	***Treatment for 5 days without stimulation***				
		%G0/G1	*%S*	*%G2/M*	*%S+G2/M*	
1.	Cellular control	89.44	3.15	7.41	10.56	100.0
2.	fraction 33 from mixture A	89.6	3.85	6.55	10.4	110.17
3.	fraction 34 from mixture A	88.77	5.38	5.85	11.23	138.81
4.	Calcitonina 5ng/ml	78.82	12.13	9.05	21.18	135.94

Vitamin C and protein complexes stimulate proliferation in osteoarthritic osteoblasts in basal conditions, instead, after stimulation with calcitriol that increases the division rate, their action manifests itself in the sense of rebalancing cellular turnover and decreasing the percentage of cells in mitotic phases. Thus, the compounds active in amplifying proliferation in basal conditions counterbalance the stimulatory effect on cell division induced by calcitriol, restoring proliferative homeostasis and preventing aberrant cell multiplication. At the level of normal human osteoblasts, the protein complexes moderately induce proliferation, acting especially in the S phase of DNA multiplication

Conclusions

This study confirm a new source of proteases, such as trypsin proteinase and cysteine proteinase, to be identified and exploited. The ability of protein fractions to stimulate cell proliferation in both normal and osteoarthritic human osteoblasts recommend it as a bioactive complex in the pharmaceutical industry, alone or in association with other active substances, improving the treatment of osteoarticular diseases. It could also be noticed that a new raw material can be capitalized, with a reduced environmental footprint.

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