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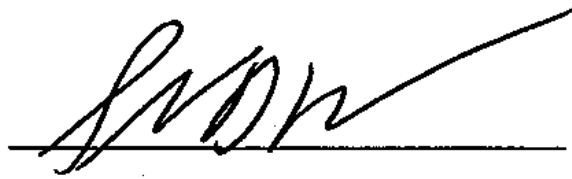
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**Characterization of a Membrane Bound Proteolytic Enzyme of
Rat Osteoblasts**

Anthony M. Orrico

**Submitted in partial fulfillment of the requirements for the degree of
Master of Science in Biology from the Department of Biology of
Seton Hall University
August , 1999**

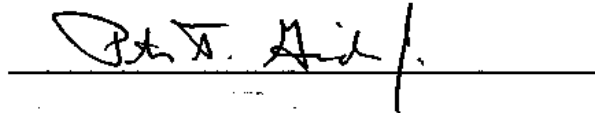
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Department Chairperson

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CHARACTERIZATION OF A MEMBRANE BOUND PROTEOLYTIC ENZYME
OF RAT OSTEOLAST

ANTHONY M. ORRICO

Abstract

A proteolytic enzyme found in the rat osteoblastic osteosarcoma cell line ROS 17/2.8 is capable of cleaving a peptide substrate for protein kinase C-mediated phosphorylation (PSPKC, Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys). This activity cleaves the Ser-Arg, Thr-Leu, and Ser-Val peptide bonds of PSPKC. This activity has now been purified by liquid column chromatography using diethylaminoethyl anion exchange resin and hydroxyapatite resin. The purified enzyme has a molecular weight of approximately 105 Kdaltons as determined by SDS-PAGE and demonstrates characteristics consistent with those of a matrix metalloprotease.

Introduction

Bone tissue is not usually considered a dynamic tissue, even though the skeletal system does undergo a continually rejuvenating process called remodeling (as reviewed by Teitelbaum 1990). Two distinct cell types working in concert, the osteoblasts and the osteoclasts accomplish remodeling. Osteoblasts, which are mesenchymally-derived cells, synthesize the organic components of the bone matrix. Earlier reports have demonstrated that type I collagen (Menanteau et al., 1982), osteocalcin (Hauschka et al., 1975; Price et al., 1976), osteonectin (Termine et al., 1981), matrix gla protein (Otagawa and Price, 1986), and bone sialoprotein I and II (Fisher et al., 1983) are all bone matrix proteins that are synthesized by osteoblasts. Defined regions of mineralized bone are resorbed by cells derived from the monocyte lineage, the osteoclasts. In earlier studies (Malone et al., 1982, Mundy and Poser, 1983) it was suggested that osteocalcin

demarcated regions to be resorbed. After resorption, osteoblasts synthesize proteins, mainly type I collagen, which fills the resorbed area. This newly repaired area, called the osteoid, at least partly mineralizes via the enzymatic activity of alkaline phosphatase (as described by Puzas 1986). Osteoblasts also synthesize a number of proteases in addition to the proteins of the osteoid, at least one of which, collagenase, has been suggested in earlier studies by Heath et al. (1984) and Otsuka et al. (1984) to be involved in the remodeling process. It had been demonstrated in a number of studies (van Leeuwen et al. 1992; Reinhardt and Horst, 1992; Vargas et al., 1989; Koch et. al., 1992) that protein kinase C (PKC) is an important signaling pathway for different effectors of osteoblastic function via the phosphorylation of different substrate proteins. It was observed that during the course of examining the potential role of PKC on osteoblast gene expression, the rat osteoblastic osteosarcoma cell line ROS 17/2.8 and other osteoblast cells synthesize proteolytic activities which are capable of cleaving a peptide substrate for PKC-mediated phosphorylation (PSPKC). The lack of activators required for activity, the lack of effect of various inhibitors on the activities, the pH optima, and the substrate specificity demonstrated that there are two new, distinct proteolytic activities (Guidon and Harrison, 1996). Pefabloc, a serine protease inhibitor, inhibits both activities and also EDTA inhibits one of the activities. EDTA has been shown to inhibit activities in matrix metalloproteinases. It was also shown that the protease inhibitors pepstatin, bestatin, E-64, leupeptin and phosphoramidon do not block either of these proteolytic activities.

In order to further characterize the proteolytic activities, the peptide bonds which were hydrolyzed by the activities were characterized. Guidon, Perrin, and Harrison (1996) used gel electrophoresis to determine the identity of the peptides that were generated when the proteolytic activities cleaved PSPKC.

While preliminary characterization of the proteolytic activities had been reported (Guidon and Harrison, 1996, Guidon, Perrin, and Harrison, 1996), identification of the proteolytic activities had yet to be established. Enrichment/ purification of the activities needed to be accomplished to further understand these activities and their potential role in osteoblast metabolism.

In modern enzymology, affinity chromatography has become the method of choice for the purification of macromolecules. This method exploits the unique specificity found in biological systems for the purification of enzymes, coenzymes, enzyme inhibitors, hormone receptors, lectins, nucleic acids, and antibodies. In principle, affinity chromatography can be used for the isolation of either component of a reversibly interacting biological system (Chin-An Hu, 1984). The basic requirement is the immobilization of one of the components (ligand) on an insoluble support in such a way that its specific binding activity is retained. The support with immobilized ligand is packed into a chromatographic bed. After a crude tissue extract has been applied to the column, the protein that specifically interacts with the ligand is retained, while other proteins, having no

affinity for the immobilized ligand, are eluted. The retained protein can be eluted specifically from the column either by adding an excess of ligand to the equilibrating buffer or by using substances such as salts or denaturants that interfere with the protein-ligand interactions. The success of affinity chromatography depends largely on how closely the conditions used in the experiment mimic the native or biological interactions. For the successful operation of affinity chromatography, a proper selection of the insoluble support should be relatively hydrophobic, stable to chemical reagents, resistant to microbial and enzymatic decomposition, and readily coupled to the ligand. Careful consideration should also be given to the steric restriction imposed by the immobilization of ligand. Steric effects can be overcome by using spacers; however, both matrix and spacer should lack nonspecific absorption sites (Smith et al., 1983). The other important factor is the selection of a ligand. There are two classes of ligands: general and specific. General ligands (i.e. NAD, AMP, Cibacron Blue, and lectins) interact with many different proteins. The ligands that interact with one or a few proteins (e.g. antigens) are called specific ligands.

The goal of this thesis was to substantially enrich/ purify the proteolytic activities found in the rat osteoblast cell line ROS 17/2.8 capable of cleaving the peptide substrate for PKC-mediated phosphorylation, PSPKC. Potential physiological roles for these activities will be discussed.

Materials and Methods

Cell Culture

The ROS 17/2.8 osteoblastic osteosarcoma cell line used in this study was characterized by Majeska et al. (1980) and received from Dr. R. Majeska (Mount Sinai Medical College, New York, NY), via the laboratory of Dr. R. Blockman (The Hospital for Special Surgery, New York, NY). ROS 17/2.8 cells were maintained in Ham's medium supplemented with 10% newborn calf serum, 2.5 mM L-glutamine, 1.1 mM Ca Cl₂, 28 mM HEPES, and 50 µg/ml penicillin/streptomycin. Cells were grown in 75 cm² Falcon flasks and then subcultured into Falcon 6 well plates for the experiments described here. After the cells were grown to confluence the monolayers were harvested for assay of proteolytic activity. For the purification studies described here, cells were grown in the absence of newborn calf serum to avoid the presence of bovine serum albumin, an abundant protein found in the serum which is capable of bridging to the cell membrane.

Reagents

A PepTag non-radioactive protein kinase assay kit for PKC and molecular biology grade urea were purchased from Promega Corporation (Madison, WI). Other peptides used in this study were purchased from Sigma Biochemical Co.

(St. Louis, MO). These peptides were labeled at the N-terminal end using 7-hydroxy-4-methylcoumarin-3-acetic acid, succinimidyl ester from Molecular Probes (Eugene, OR) according to the manufacturer's protocol. A protease inhibitor kit was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

Proteolytic Activity Assay

ROS 17/2.8 cells were lysed in a hypotonic lysis buffer originally designed to allow for the measurement of PKC activity as described by Boyle et al. Al. (1991). This buffer consisted of 20mM HEPES-NaOH (pH 7.4), 5mM KCl, 5mM MgCl² 5mM DTT, 0.1 mM EDTA, 50 mM NaF, and 0.1% NP-40. Typically, one well of a 6-well Falcon plate was harvested with 200 μ l of hypotonic lysis buffer. Ten microliters of the cell lysate (approximately 4 μ g protein, as determined using Protein Determination Reagent, United States Biomedical Corp., Cleveland, OH) was then incubated with 5 μ l PKC peptide (2 μ g) and 10 μ l ddH₂O for 30 minutes at 30°C to assay for proteolytic activity. After incubation, the samples were boiled for 10 minutes, then loaded onto a 1.0 % agarose gel in 50 mM Tris buffer, pH 8.0. The samples were then subjected to electrophoresis for 30 minutes at 100 V, after which the samples in the gel were visualized using ultraviolet light and photographed with Polaroid Type 665 film.

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide-SDS gels (final concentrations: 7.5% acrylamide, 0.375 M Tris, pH 8.8, 0.1% SDS) were prepared in a Mini-Protean SDS PAGE apparatus (Bio-Rad Laboratories, Hercules, CA). Samples were diluted with 2x sample buffer and the samples were boiled for 2 minutes at 100 °C. The samples were subjected to electrophoresis at a constant voltage of 100 V until the dye front reached the end of the stacking gel (approximately 45 min.) and then 200 V until the dye front neared the bottom of the gel (approximately 1 hr). Protein bands in the gels were visualized using either Coomassie blue or silver stain.

Liquid Column Chromatography

The first step of column chromatography purification used the DEAE (Diethylaminoethyl) Bio-Gel Agarose from Bio-Rad Laboratories. An Econo-column was packed with 2 ml of the gel matrix slurry and 10mM Tris/pH 7.4 1mM EDTA was used to equilibrate the matrix at the maximum flow rate of 1 ml/min. ROS 17/2.8 cells were harvested from Falcon culture dishes using 1x cell culture lysis buffer (Promega , Inc, Madison, WI). The liquid lysate was collected using a Pasteur pipette, and the remaining cell membranes which remained adhered to the culture dishes were washed once in PBS, and then collected in PBS by scraping the plate with a rubber policeman. The sample was clarified by centrifugation (14000xg for 10 min), and the pelleted fraction was applied to the

DEAE column. After loading the sample, the column was washed with Tris-EDTA buffer and eluted with five 2.5 ml aliquots of 0.1 M, 0.2M, 0.3M, 0.4M, and 0.5M NaCl. Samples were collected and tested for the enzyme using the proteolytic activity assay. Those samples that contained the activity were pooled and applied to a ceramic hydroxyapatite resin (Bio-Rad). The column matrix was packed and equilibrated identically to the first chromatographic step, but was eluted with 2.5ml aliquots of 0.05 M, 0.1 M, 0.15M, 0.2 M, 0.25 M, 0.3 M, and 0.5 M sodium phosphate buffer. The samples were once again pooled based on results of the proteolytic activity assay.

Results

Observation of Proteolytic Activities in ROS 17/2.8 Cell Lysates

PSPKC is an N-terminal, fluorescently labeled peptide (P-L-S-R-T-L-S-V-A-A-K), which is a specific substrate for phosphorylation by protein kinase C (PKC).

When not phosphorylated, PSPKC has a net charge of +1 at pH 8.0; the arginine and lysine residues each contribute a +1 charge, while the free COO-group of the C-terminal amino acid contributes a -1 charge. After phosphorylation of the threonine residue by PKC, the net charge of PSPKC can easily be determined by electrophoresis in a 1.0% agarose gel in 50 mM Tris buffer, pH 8.0.

To examine how various effectors may influence signal transduction pathways in ROS 17/2.8 cells, studies were aimed at identifying protein kinase C (PKC) activity in this cell line. When a ROS 17/2.8 cell lysate was incubated with PSPKC for 30 min. PKC activity itself was not detected; rather, two proteolytic digestion products were observed (Figure 1). One of the products possessed a net charge of 0 (band B). This band was generated by the proteolytic cleavage of PSPKC, which cut between the terminal lysine residue and the C-terminal side of the arginine residue. In addition, a second peptide fragment possessing a -1 net charge (band C) was also generated as a result of PSPKC incubation with the ROS 17/2.8 cell lysate. This cleavage event had to occur to the N-terminal side of the arginine residue. Detection of the fragment having a net charge of 0 always preceded detection of the fragment having a net charge of -1. Boiling the ROS 17/2.8 cell lysate prior to incubation with PSPKC resulted in a loss of proteolytic activity.

Purification with BSA Deficient Cells

SDS-PAGE of cells grown in the presence and absence of serum (Figure 2) shows that a large amount of albumin can be found associated with cells grown in the presence of serum. Presence of this abundant protein would only complicate the purification of the proteolytic activities. If omission of the fetal bovine serum in the culturing of the cells did not affect the proteolytic activities,

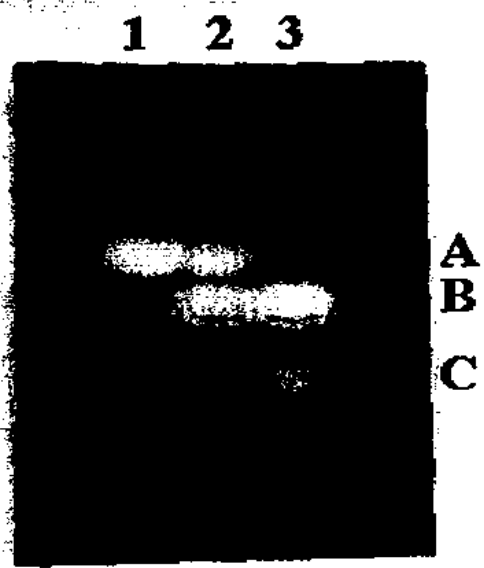


Figure 1 Proteolytic Activity of ROS 17/2.8 cells on PSPKC. PSPKC (lane 1) was incubated with a ROS 17/2.8 extract for 30' (lane 2) or 60' (lane3). The reaction was then separated using a 1% agarose gel in 50 mM Tris pH 8.4. Band A is intact PSPKC, band B has a net charge of 0, and band C has a net charge of -1.

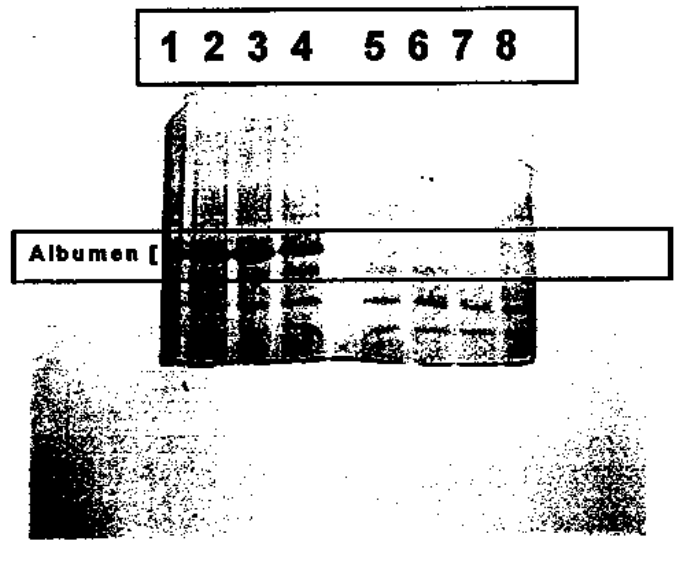


Figure 2 SDS-PAGE shows the large amount of albumin in cells grown in the presence of serum. Lanes 1-4 were cultured in serum and Lanes 5-8 were cultured without serum.

then growing cells under serum free - conditions would facilitate the purification efforts. As can be seen in Figure 3, there was no difference in the amount of protein activity in cells cultured in the absence or presence of serum, therefore cells were grown in the absence of serum prior to harvesting the cells for purification of the proteolytic activities.

Determination of Location of Proteolytic Activity in Cellular Material

In order to initially fractionate, on a cellular level, the location of the proteolytic activities, determination of whether the activities were membrane-associated or cytoplasmic had to be identified. A reporter lysis buffer that lysed the cell membranes but kept them attached to the T-75 flask was used for the fractionation studies. The cytoplasmic fraction and membrane fraction were collected after lysis and subjected to the proteolytic activity assay (Figure 4). The reporter supernatant sample showed no signs of enzymatic activity (lane 4) where as abundant activity was seen in the membrane fraction (lane 5).

DEAE Column Chromatography

The DEAE anion exchange resin was the first step in the purification of the enzyme. Cell lysates enriched for membrane fractions (1.8ml) were loaded onto a 1 ml column equilibrated with equilibration buffer (10mM Tris, 1mM EDTA, pH

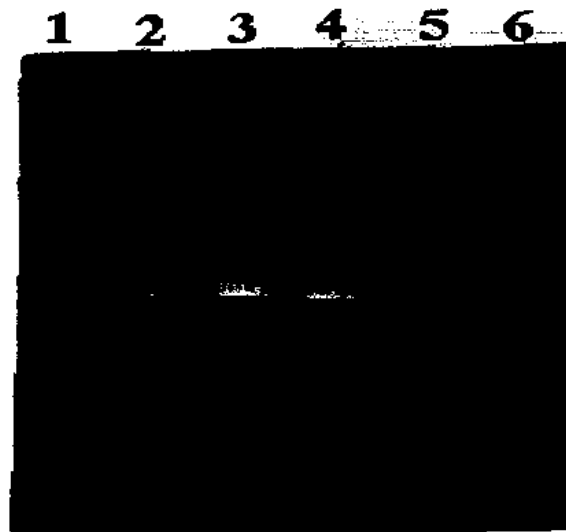


Figure 3 Absence of serum in growth media does not affect the amount of proteolytic activity in ROS 17/2.8 cells. ROS 17/2.8 cells grown in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of serum were assayed for proteolytic activity. ROS cell lysates were incubated with PSPKC for 0' (1, 2), 30' (3, 4), and 60' (5, 6). The reactions were then separated on a 1% agarose gel in 50 mM Tris pH 8.0.

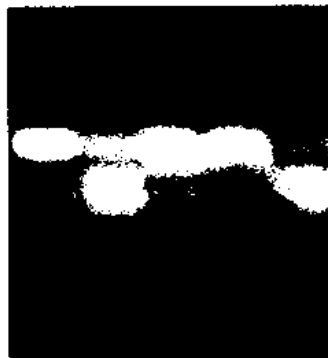


Figure 4 Proteolytic activity assay of the cytoplasmic and membrane fractions of the ROS cells collected after lysis. Lane 1 – Neg. Control; Lane 2 – Pos. Control; Lane 3 – ROS cells boiled; Lane 4 – Supernatant (cytoplasmic) fraction; Lane 5 – Pellet (membrane) fraction.

7). After 1.5 ml had flowed through the system 0.5 ml fractions were collected, the equilibration buffer (2.5 ml) was applied next to ensure that any protein that was not bound would not taint the first elution fractions. The column was then eluted with 2.5 ml aliquots of the following eluents: 0.1M NaCl, 0.2M NaCl, 0.3M NaCl, 0.4M NaCl, and 0.5M NaCl. The third fraction of each elution was tested for proteolytic activity (Figure 5). Activity, and presumably the protein of interest, was found in fraction 3 of the 0.2M elution. To distinguish which exact fractions contained the protein another proteolytic assay was performed on the first fraction from the 0.1M NaCl elution to the first fraction of the 0.3M NaCl elution (Figure 6). After these results, the second fraction of the 0.2M NaCl to the first fraction of the 0.3 M NaCl were pooled together and would be used as the load for the next chromatography step.

Ceramic Hydroxyapatite Column Chromatography

The Biorad Macro-Prep Ceramic Hydroxyapatite resin was the second and final step in the purification of the enzyme. The pooled fractions from the DEAE column (3.5 ml) were diluted to 10 ml with equilibration buffer (10mM Tris 1mM EDTA pH 7) to reduce the NaCl concentration to approximately 0.06 M. One ml of the matrix resin was equilibrated with 5 ml equilibration buffer, after which time the sample was applied. Two and a half milliliters of equilibration buffer were applied to wash out any protein that was not bound specifically to the column matrix and as a result would not taint the first elution fractions. The column was

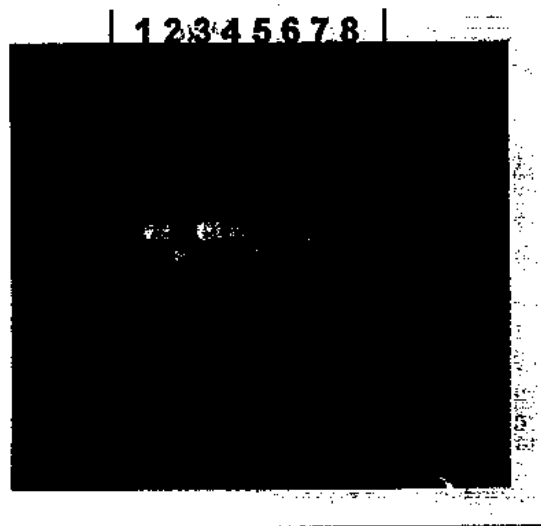


Figure 5 Agarose gel of 3rd fraction of each NaCl elution performed on the DEAE matrix used to test for proteolytic activity. Lane 1 - Neg. Control; Lane 2 - DEAE Load; Lane 3 - Equilibration Buffer Flowthrough; Lane 4 - 0.1M NaCl; Lane 5 - 0.2M NaCl; Lane 6 - 0.3M NaCl; Lane 7 - 0.4M NaCl; Lane 8 - 0.5M NaCl.

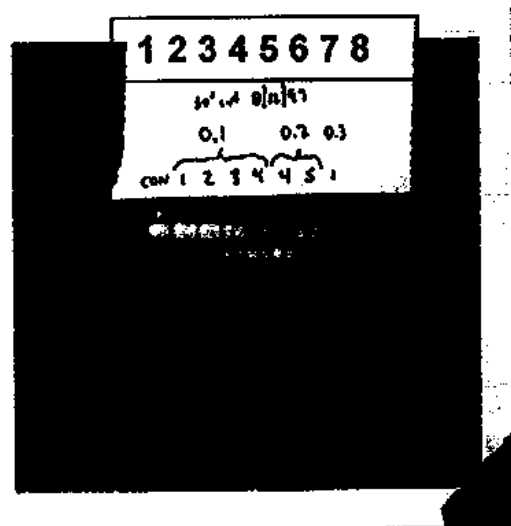


Figure 6 Agarose gel to distinguish which exact fractions exhibit proteolytic activity. Lane 1 - Neg. control; Lanes 2-5 - 0.1M NaCl fractions 1-4; Lane 6 - fraction 4 of 0.2M NaCl; Lane 7 - fraction 5 of 0.2M NaCl; Lane 8 - fraction 1 of 0.3M NaCl.

then eluted with 2.5 ml of the following eluents: 0.05M, 0.1M, 0.15M, 0.2M, 0.25M, 0.3M, and 0.5M sodium phosphate. The column was then regenerated with 2.5 ml of 1M sodium phosphate to remove the most tightly bound proteins. The third fraction of each elution was tested for proteolytic activity (Figure 7). Activity, and therefore the protein, was found in fraction 3 of the 0.05M elution. To distinguish which exact fractions contained the protein; another proteolytic assay was performed on the third fraction from the equilibration wash to the second fraction of the 0.1M sodium phosphate elution (Figure 8). Based on these results, the fifth fraction of the equilibration wash to the fourth fraction of the 0.05 M sodium phosphate elution were pooled together.

SDS-PAGE Analysis

To illustrate the efficacy of each of the resins in the purification of the protein activity, the protein profile of the crude lysate, the DEAE pooled fractions, and the hydroxyapatite pooled fractions were determined using a 4-15% gradient SDS-PAGE and silver staining (Figure 9). The single predominant band in lane 4, with an approximate MW of 105,000 daltons, is the best candidate to possess the proteolytic activity.

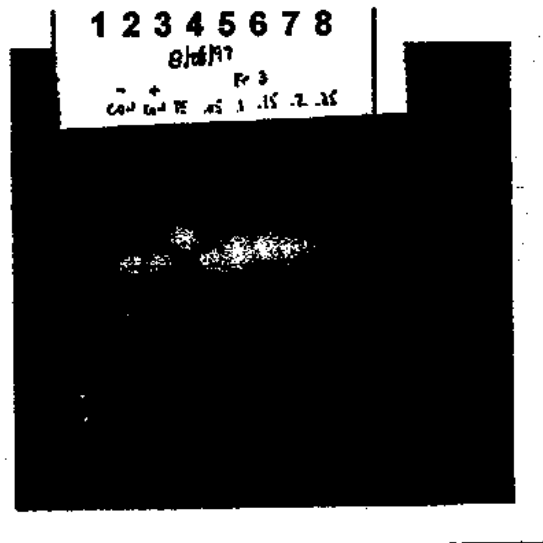


Figure 7 Agarose gel of 3rd fraction of each Sodium Phosphate elution performed on the HAP matrix used to test for proteolytic activity. Lane 1 - Neg. Control; Lane 2 - HAP Load; Lane 3 - Equilibration Buffer Flowthrough; Lane 4 - 0.05M; Lane 5 - 0.1M; Lane 6 - 0.15M; Lane 7 - 0.2M; Lane 8 - 0.25M.

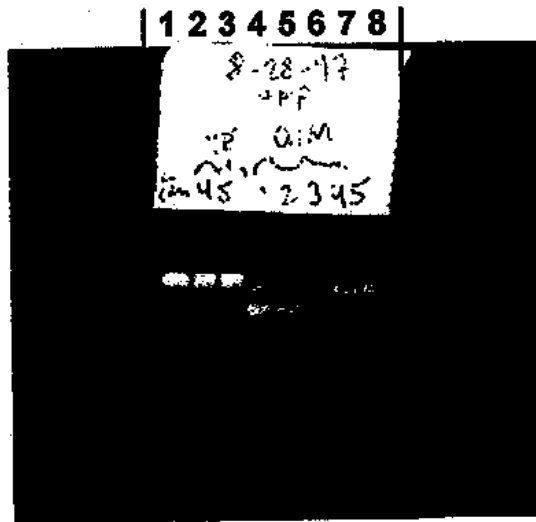


Figure 8 Agarose gel to distinguish which exact fractions exhibit proteolytic activity. Lane 1 - Neg. control; Lane 2 - fraction 4 of Equilibration flowthrough; Lane 3 - fraction 5 of Equilibration flowthrough; Lanes 4-8 - 0.1M Sodium Phosphate fractions 1 through 5.



Figure 9 SDS-PAGE Analysis of the enrichment of the PSPKC proteolytic activity in ROS 17/2.8 cells visualized with silver stain. Lane 1 - A MW marker; Lane 2 - ROS cell lysate applied to the DEAE column; Lane 3 - DEAE enriched material applied to the HAP column; Lane 4 - Isolated proteolytic activity.

Molecular Weight Filtration

To confirm the identity of the 105 Kd protein as the proteolytic activity, the hydroxyapatite pooled material was subjected to an Amicon microfiltration system with a molecular weight cutoff of 100,000 daltons. The retentate fraction and the flow through material were tested for protein activity. The retentate showed activity and the filtrate did not indicating that the protein was greater than 100 Kd in size (Figure 10).

Discussion

The protein purification protocol described here was directed by research already conducted on this enzyme. The preferred peptide bond cleavage site is one property by which proteolytic enzymes may be identified and determination of substrate specificity for the proteolytic activity is another. The peptide fragment of PSPKC with a net charge of -1 generated after incubation with the ROS 17/2.8 cell lysate was identified as Pro-Leu-Ser, and the two peptide fragments with a net charge of 0 were Pro-Leu-Ser-Arg-Thr and Pro-Leu-Ser-Arg-Thr-Leu-Ser. From the data collected in the current study, it appears that a single proteolytic activity is responsible for generating both peptide fragments.

The protease activities described were inhibited in pre-incubation with EDTA, a known inhibitor of matrix metalloproteases. Metalloproteases (MMPs) contain

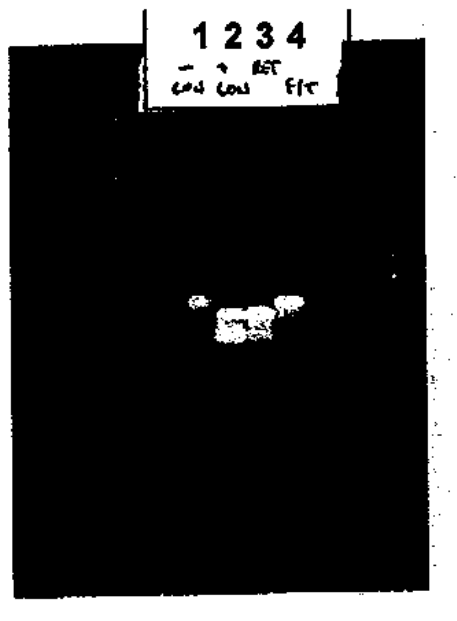


Figure 10 Agarose gel indicating the retentate from the 100,000 dalton filter exhibited proteolytic activity. Lane 1 - Neg. Control; Lane 2 - Pos. Control; Lane 3 - 100,000 dalton filter retentate; Lane 4 - 100,000 dalton filter flowthrough.

the consensus active-site sequence HEXGHNLGXXHD. Structural analysis suggests that the three histidines (underlined) bind zinc, the glycine (*italicized*) allows a turn, and the glutamic acid (**bold**) is the catalytic residue. While early work on MMPs focused on the secreted form of this family of enzymes, metalloprotease domains have been found in a new class of membrane proteins called ADAMs, A Disintegrin And Metalloprotease domain (Wolsberg et al., 1995). ADAMs have been found in various roles: spermatogenesis, in sperm binding and fusion, and in adhesive and proteolytic reactions in nonproductive tissues (Wolsberg et al., 1995).

Distinctions have been made between integral transmembrane proteins on the basis of their orientation and the number of times the protein spans the lipid bilayer. Type I proteins are synthesized with a cleavable N-terminal signal peptide which is removed early in biosynthesis, but they are retained in the membrane by a hydrophobic sequence of amino acids. In the case of Type II cell-surface proteins the membrane anchor is the uncleaved signal peptide and the protein is oriented with a short, hydrophilic cytoplasmic domain and with the bulk of the protein, including the C-terminus, facing the extracellular space. Some single membrane-spanning proteins, especially those involved in intracellular signaling processes, exhibit a 'dumb-bell' structure in which the transmembrane segment is towards the middle of the protein sequence such that a significant proportion of the protein is exposed at both membrane surfaces.

Finally, a wide range of multiple membrane-spanning proteins exist (Type III), ranging from two transmembrane segments in the case of subunit c of F₀-ATPase complex through to 24 in the case of the sodium channel in neurons. In the majority of cases it is likely that the cleavage occurs at a single, unique site defined by the specificity of the protease and the topology of the protein substrate (Kini et al, 1996).

A diverse range of membrane proteins of Type I or Type II topology also occur in a circulating, soluble form. These soluble forms are often derived from the membrane form by proteolysis by a group of enzymes referred to collectively as 'secretases' or 'shedases'. The cleavage generally occurs close to the extracellular face of the membrane, releasing physiologically active protein. This secretion process also provides a mechanism for down-regulating the protein at the cell surface. Examples of such post-translational proteolysis are seen in the Alzheimer's amyloid precursor protein, the vasoregulatory enzyme angiotension converting enzyme, transforming growth factor-alpha, the tumor necrosis factor ligand and receptor superfamilies, certain cytokine receptors, and others. Since the proteins concerned are involved in pathophysiological processes such as neurodegeneration, apoptosis, oncogenesis and inflammation, the secretases could act as novel therapeutic targets. Recent characterization of these individual secretases has revealed common features, particularly sensitivity to certain metalloprotease inhibitors and up-regulation of activity by phorbol esters. It is therefore likely that a closely related family of metallosecretases controls the

surface expression of multiple integral membrane proteins. Current knowledge of how the various secretases control the surface expression of multiple integral membrane proteins is not known (Hooper et al., 1997).

Recent evidence has indicated that disintegrins and metalloproteases are derived by proteolysis of a common precursor protein (Kini and Evans, 1992). This precursor protein was proposed to contain four distinct structural domains. Based on an analysis of the putative start codon, signal peptide and the region upstream of the start codon in the cDNA sequence of the precursors, it was proposed that C-type lectin related proteins may also be derived from disintegrin metalloprotease precursors (Kini and Evans, 1996). Proteolytic cleavage in interdomain segments results in the formation of disintegrins, metalloproteases, CLRPs, and other proteins with unknown biological properties. During the process, some of the interdomain peptides will be released. It has not been determined if this region of the molecule contributes to the biological role of the precursors or the products derived by the proteolysis of the precursors.

After demonstrating that the proteolytic activity was found in the membrane with a molecular weight of approximately 105 Kdaltons, it is felt that the protein may be a member of the ADAM family. Consistent with this hypothesis is that the activity is inhibited by EDTA, a known metalloprotease inhibitor. Subsequent characterization of this enzyme would include purifying sufficient quantities of the enzyme to obtain amino acid sequence data, which would allow further

characterization of this enzyme. Isolation and characterization of novel proteases from osteoblast cells may provide key insights into previously unknown protein processing pathways in these cells and mineralized tissues.

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