Purification and Characterisation of a Human Procollagen C-proteinase

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ABSTRACT

Procollagen C-proteinase is an extracellular zinc metalloprotease, which is expressed at low levels in connective tissue. By cleaving the C-propeptide from the soluble precursor procollagen, C-proteinase plays a pivotal role in the formation of collagen fibrils in normal and fibrotic connective tissue, and despite a wealth of knowledge on collagen biosynthesis, little is known about this key enzyme. The pharmacological treatment of these fibroproliferative diseases has been limited and a novel approach by inhibiting C-proteinase activity is currently being investigated. In these studies C-proteinase was partially purified from leg tendons from 400 dozen chick embryos, using Green A DyeMatrex, concanavalin A-Sepharose, and heparin-Sepharose chromatography, and Sephacryl S-300 gel filtration. The C-proteinase was contaminated with a number of other proteins and attempts at obtaining a N-terminal sequence were unsuccessful, so other sources of enzyme were examined.

Here the partial purification of human C-proteinase, from MG63 osteosarcoma cells, is described. Media were generated using mass cell culture, and levels of C-proteinase were 1.2 fold greater than that in the chick leg tendon organ culture media. Two forms, E-A and E-B, of enzyme were separated, in a ratio of 33:1, where the E-B form was purified with the C-proteinase enhancer molecule. The two forms were possibly a result of different levels of glycosylation, or that one was an alternatively spliced product of the other, or that this C-proteinase exists as a precursor molecule.

This C-proteinase was a zinc metalloprotease, dependent on 5-10 mM $CaCl_2$ for activity. It was shown to cleave at the -Ala-Asp- bond in both proα1(I) and proα2(I) chains in human and chick procollagen. In cell culture, levels of C-proteinase were significantly upregulated by TGFβ1, without a comparable increase in deposited collagen levels, suggesting that TGFβ1 acts by increasing C-proteinase levels.

Two dimensional cyanogen bromide cleavage peptide patterns showed that E-A and E-B were identical to the human 80 kDa bone morphogenetic protein-1, confirming this recent, exciting and significant discovery. This relates an enzyme involved in matrix formation to a protein related to gene products, known to be involved in embryogenesis. Functionally, human C-proteinase and BMP-1 proteins are very different, and how they are linked is unknown. This potentially opens up an exciting field of research into understanding the association of these proteins, and in the development of inhibitors, and eventual treatment of fibrosis.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

NOTES ON COPYRIGHT AND OWNERSHIP

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General

α chains	the monomeric subunits of collagen
BSA	bovine serum albumin
BB	British Biotech Pharmaceuticals Ltd. (Oxford, England)
BMP	bone morphogenetic protein
C-	carboxyl
°C	degree centigrade
^{14}C	radioactive isotope of carbon
CAPS	(3-[cyclohexylamino]-1-propanesulfonic acid
CET	chick embryo tendons
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CNBr	cyanogen bromide
CO_2	carbon dioxide
C-proteinase	procollagen C-proteinase
CUB	Complement-Uegf-BMP-1
D	axial periodicity characteristic of collagen fibrils
DEAE-cellulose	diethylaminoethyl cellulose
dpp	decapentaplegic
Dr	*Drosophila*
DS	dextran sulphate
DMEM	Dulbecco's modified Eagle's medium

E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino) butane
E-A	a 98 kDa C-proteinase form
E-B	a 96 kDa C-proteinase form
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol -bis(oxy-ethylenenitrilo)tetraacetic acid
ECM	extracellular matrix
FAF	fibroblast activating factor
FCS	foetal calf serum
FGFb	fibroblast growth factor basic
g	force of gravity
Gal	galactose
Glc	glucosamine
$HClO_4$	perchloric acid
hr	hour
Hu	human
IL-1β	interleukin-1-*beta*
IL-4	interleukin-4
kDa	kilodalton
L	litre
LSC	liquid scintillation counts
MMP	matrix metalloproteinase

μCi	microCurie
μg	microgram
μl	microlitre
M	molar
Man	mannose
mg	milligram
Mg^{2+}	divalent magnesium ion
MG63	human osteosarcoma cells
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mTld	mammalian tolloid-like
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N-	amino
NaN_3	sodium azide
N-proteinase	procollagen N-proteinase
OH	hydroxyl
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pC-collagen	an intermediate in the normal processing of procollagen to collagen containing the C-propeptides but not the N-propeptides

PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEG	polyethylene glycol
pN-collagen	an intermediate in the normal processing of procollagen to collagen containing the N-propeptides but not the C-propeptides
PMSF	phenylmethylsulfonyl fluoride
proα chains	the monomeric subunits of procollagen
PVDF	polyvinylidene difluoride
SDS	SDS
sec	second
SEM	standard error of the mean
TEMED	N, N, N', N'-tetramethylethylenediamine
TGFβ	transforming growth factor-beta
TIMP	tissue inhibitor of matrix metalloproteinase
Tld	tolloid
Tlr-1	tolloid-related-1
TNF	tumor necrosis factor
TRE	transcription responsive element
Tris	Tris-(hyrdroxymethyl)aminomethane
U	units
v/v	volume to volume
w/v	weight to volume

Amino Acid Abbreviations

Alanine	Ala	A
Asparagine or Aspartate	Asx	B
Cysteine	Cys	C
Aspartate	Asp	D
Glutamate	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V

Tryptophan	Trp	W
Tyrosine	Tyr	Y
Glutamine or Glutamate	Glx	Z
Hydroxylysine	Hyl	J
Hydroxyproline	Hyp	O

I dedicate this thesis to my mother, and the rest of my family

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1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Little is known about procollagen C-proteinase, an enzyme central and critical in collagen fibril formation in connective tissues, and so, current research has focused on understanding this key enzyme. There are a number of diseases, and pathological conditions in which the excessive synthesis and deposition of fibrillar collagen leads to the destruction of normal tissue architecture and function. These diseases include debilitating disorders such as liver cirrhosis, pulmonary fibrosis, rheumatoid arthritis, and hypertrophic dermal and keloid scarring. Attempts to reduce collagen deposition have mainly focused on inhibiting prolyl 4-hydroxylase, a critical enzyme in the biosynthesis of procollagen. However, it has proved difficult to design potent inhibitors that can be delivered in sufficient concentration to inhibit this enzyme which is located in the endoplasmic reticulum.

Procollagen C-proteinase, however, catalyses the rate limiting and committed step in the extracellular conversion of soluble procollagen to insoluble collagen. This enzyme is now generally accepted to be a prime target for therapeutic intervention in the treatment of fibrosis and related disorders characterised by ectopic and excessive collagen deposition. This thesis examines the purification and biochemistry of a human C-proteinase.

1.2 THE FIBRILLAR COLLAGENS

Collagens in the form of multimeric fibrils and associated proteins, are the major components of connective tissue in animals, providing structural support for the tissues, organs and cells. Fibrillar collagen provides mechanical strength in bone and cartilage, tensile strength in tendons and ligaments, compartmentalisation and protection in the dermis and cornea, and specialised properties in the glomerular basement membrane. There are twenty genetically distinct collagens, classified either as fibrillar (fibril-forming) or non-fibrillar collagens (for reviews see Vuorio & de Crombrugghe, 1990; Miller & Gay, 1987). At least five fibrillar collagens, types I, II, III, V and XI, are known which exist as highly organised D-periodic fibrils and fibres in the ECM in vertebrates (Table 1.1) (for review see Kadler *et al.*, 1996). These collagens comprised of semi-rigid rod shaped triple-helical molecules are the major structural components of cross-striated collagen (Kuhn, 1987).

Each collagen molecule is composed of three left handed helical chains (α chains), coiled around each other forming a right handed super helix, approximately 1.5 nm in diameter and 300 nm in length. Each chain contains approximately 1000 amino acids, with a characteristic repeating triplet sequence Gly-X-Y, where X and Y denote any imino or amino acid other than glycine, and are frequently proline and hydroxyproline respectively (Kielty *et al.*, 1993). The small glycine residues are vital for the formation of the triple-helix, as they occupy the restricted space in the centre of the helix at every third position, forming hydrogen bonds with residues in adjacent chains. Stability of the folded molecule is further enhanced by the steric repulsion between the large cyclic

Table 1-1 The Fibrillar Collagens

Type	Molecular configuration	Supramolecular structure	Distribution
I	$[\alpha1(I)]_2\,\alpha2(I)$, $[\alpha1(I)]_3$	Large diameter 67 nm banded fibrils	Bone, tendon, skin, ligament, cornea
II	$[\alpha1(II)]_3$	Small diameter 67 nm banded fibrils	Cartilage, vitreous, notochord
III	$[\alpha1(III)]_3$	Small diameter 67 nm banded fibrils	Aorta, foetal skin, gut
V	$[\alpha1(V)]_2\,\alpha2(V)$, $\alpha1(V)\alpha2(V)\alpha3(V)$, $[\alpha1(V)]_3$	Small diameter fine fibrils	Bone, skin, ligament, placenta
XI	$\alpha1(XI)\alpha2(XI)\alpha3(XI)$	Same as type V	Cartilage, intervertebral disc

Information is composed from Kielty *et al.*, 1993 and Ayad *et al.*, 1994.

proline and hydroxyproline residues, which limits rotation (Bachinger *et al.*, 1993). Formation of extra hydrogen bonds between hydroxyproline and the backbone is critical for thermal stability, as the transition temperature of the triple-helix denaturation is proportional to the extent of proline hydroxylation. In mammalian collagens, the use of non prolyl or hydroxyprolyl residues in the X and Y position is often associated with clusters of hydrophobic and charged residues. These decrease the stability of the triple-helix, but are essential in determining how collagen molecules associate with each other, especially as a number of collagens form heterotypic fibrils (Linsenmayer, 1991).

The triple-helical conformation of collagen ensures that adjacent amino acids are buried within the interior of the molecule, conferring a high degree of resistance to attack by non-specific proteases such as pepsin and trypsin. When the molecule is thermally or chemically denatured the hydrogen bonds are disrupted, the molecule unfolds and the buried residues become susceptible to proteolysis.

1.2.1 Type I Collagen

Type I collagen, the most abundant fibrillar collagen, is found in bone in association with calcium hydroxyapatite, and in tendon, dermis, ligament, cornea and dentin. Importantly, it is synthesised in response to tissue injury and in fibrous nodules found in fibrotic disease. The molecule is a heterotrimer composed of two identical α1(I) chains and one α2(I) chain. The α1(I) chain is a 1057 amino acid product from gene COL1A1 found on human chromosome 17, and the α2(I) chain is a 1039 amino acid

product from gene COL1A2 found on human chromosome 7 (Ayad *et al.*, 1994). A homotrimer containing three α1(I) chains has also been detected in embryonic chick tendon and calvaria (Jimenez *et al.*, 1977).

1.2.2 Type II Collagen

Type II collagen is found in embryonic and adult cartilage, cornea, notochord, vitreous body, and a number of epithelially derived embryonic matrices (Fitch *et al.*, 1989). The molecule is a homotrimer consisting of three identical α1(II) chains, which are a product of the COL2A1 gene found on human chromosome 12. It exists in two forms, as an alternative splice site has been described in the N-propeptide region of the precursor molecule (Ryan & Sandell, 1990). In cartilage, type II collagen molecules occur in heterotypic fibrils in association with type XI collagen, and they are also extensively cross-linked with type IX (Mendler *et al.*, 1989).

1.2.3 Type III Collagen

Type III collagen is found in stromal connective tissues (Keene *et al.*, 1987), it comprises half of the collagen in tissues of the cardiovascular system, and occurs in heterotypic fibrils with type I collagen in stromal matrices such as dermis (Fleischmajer *et al.*, 1990). The molecule is a homotrimer composed of three identical α1(III) chains. The α1(III) chain is a product of gene COL3A1 located on human chromosome 2. Compared to types I and II collagen the α chains contain high levels of glycine (greater than 333 residues) and hydroxyproline, and the chains are cross-

linked by intramolecular disulfide bonds in both the helical and non-helical (telopeptide) regions (Jukkola *et al.*, 1986).

1.2.4 Type V Collagen

Aggregates of type V collagen are found in pericellular zones in association with basal lamina, and often as thin fibrils with type I collagen in heterotypic fibrils (Birk *et al.*, 1988). Initially two forms of type V collagen were thought to exist, the predominant form of type V collagen was a heterotrimer composed of two $\alpha1(V)$ chains and one $\alpha2(V)$ chain, found in bone, dermis, and placental membrane. The second was a homotrimer composed of three chains (Haralson *et al.*, 1980). Since then a third chain ($\alpha3(V)$) has been shown to accompany the $\alpha1(V)$ and $\alpha2(V)$ chains in the heterotrimer $\alpha1(V)\alpha2(V)\alpha3(V)$ (Niyibizi *et al.*, 1984). So it is now accepted that three chains exist, and the type V class describes a homotrimer $[\alpha1(V)]_3$ and a heterotrimer $\alpha1(V)\alpha2(V)\alpha3(V)$.

1.2.5 Type XI Collagen

Type XI collagen is a heterotrimer $\alpha1(XI)\alpha2(XI)\alpha3(XI)$ and accounts for 8-10% of collagens in most cartilage (Morris and Bachinger, 1987). It is found in heterotypic fibrils with type II and IX collagens. The $\alpha1(XI)$ and $\alpha2(XI)$ chains are closely related to the $\alpha1(V)$ and $\alpha2(V)$ chains respectively, and the $\alpha3(XI)$ chain is the same gene product as the $\alpha1(II)$ chain but is more glycosylated (Burgeson and Hollister, 1979).

1.3 BIOSYNTHESIS OF TYPE I COLLAGEN

The biosynthesis of collagen is a complex process, characterised by a number of intracellular and extracellular posttranslational modifications (for review see Olsen, 1991). The polypeptide chains are firstly modified and assembled into triple helical procollagen molecules in the intracellular environment (Figure 1.1), and after transport to the extracellular environment further modifications ensue. At least ten enzymes, specific and non-specific, modify approximately 100 amino acids, during the maturation of the soluble procollagen into stable, cross-linked fibrils (Kivirikko & Myllylä, 1985) (Figure 1.2).

1.3.1 The Importance of Procollagen

A fundamentally important feature of fibrillar collagens is that they are synthesised as soluble precursor molecules, procollagens (Grant & Jackson, 1976; Fessler & Fessler, 1978; Miller, 1985). These molecules have approximately 1.5 times the mass of their corresponding collagens found in the ECM, as each α chain exhibits globular peptide extensions at the C- and N- terminal ends, termed C- and N- propeptides, respectively (Figure 1.1). The N-propeptide, shows a considerable divergence in length and domain structure between chains and species, whereas the C-propeptide is highly homologous. Both the C- and N- propeptide are crucial for the secretion of procollagen into the extracellular environment.

The N-propeptide contains three structural domains, a cysteine rich globular domain, a short triple-helical region and a short telopeptide region that connects with the major

Figure 1.1 Type I Procollagen

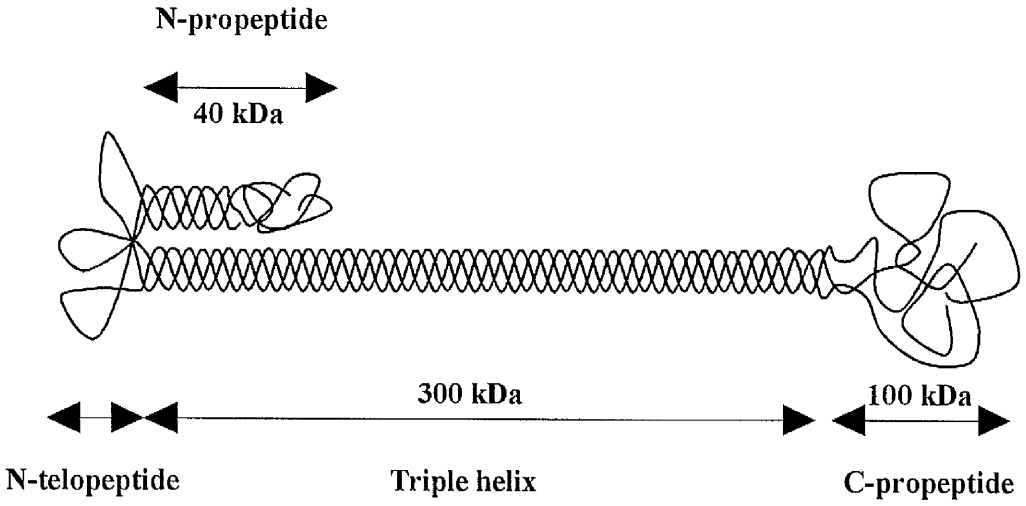
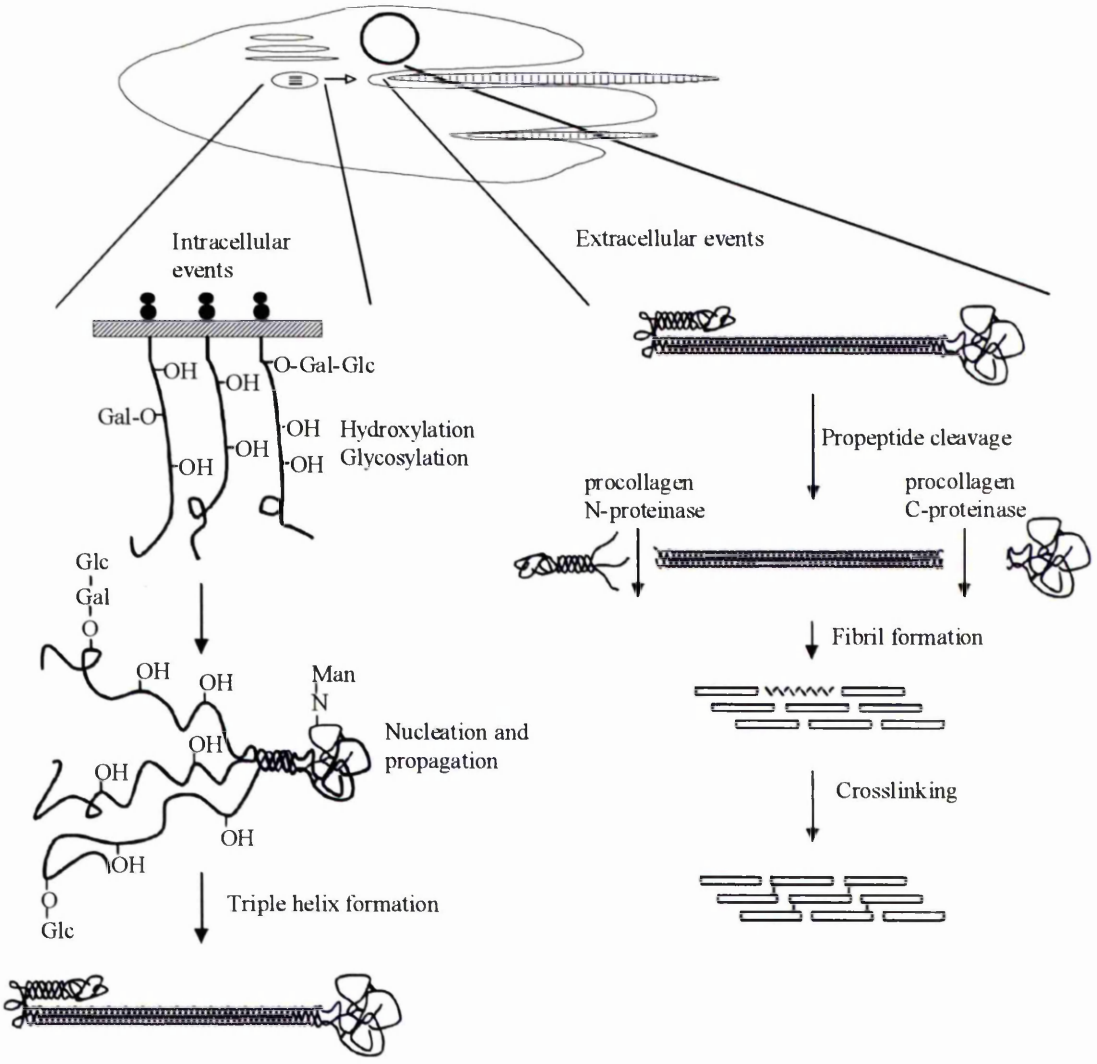


Figure 1.2 Schematic Representation of Collagen Biosynthesis



Intracellular processing of the nascent procollagen chains including hydroxylation of prolyl and lysyl residues, glycosylation of hydroxylysyl residues, triple helix formation and disulphide bond formation occurs within the cisternae of the rough endoplasmic reticulum. After secretion via the Golgi complex into the extracellular space, N- and C- proteinases cleave off the N- and C- propeptides respectively, initiating fibril formation.

triple-helix. The function of the N-propeptide is not clear but it can affect fibril morphology and, secondly, it contributes to the solubility of the precursor molecule in the intracellular environment.

The presence of the C-propeptide, however, confers greater solubility under physiological conditions on the molecule, allowing it to pass through the cell. Removal of the C-propeptide by procollagen C-proteinase lowers the solubility of procollagen approximately 10 000 fold, and is a prerequisite and necessary for the initiation of the self-assembly of collagen fibres (Kadler *et al.*, 1987). The C-propeptide consists of three peptide chains linked by interchain disulphide bonds, formed between three or four cysteine residues found close to the collagen triple-helix. Four additional cysteine residues form two intrachain disulphide bonds in each chain, which stabilises the globular structure. The C-propeptide domain, unlike the N-propeptide, exhibits the highest degree of sequence similarity between the fibrillar procollagen types and species (Kimura *et al.*, 1989). Most importantly, the location of the cysteine residues and surrounding residues are strictly conserved. It is postulated that formation of the correct disulphide bridges ensures the correct registration of the three α chains prior to helix assembly (Schofield & Prockop, 1973).

1.3.2 Pre-procollagen mRNA Translation

All pre-procollagen mRNAs are translated on free ribosomes to polypeptide chains, with a highly homologous, hydrophobic signal sequence at the N-terminal end. The signal peptide, similar to those in most other secreted peptides (Gierasch, 1989) forms

a complex with a signal recognition peptide (SRP). This SRP/polypeptide complex then associates with either, a receptor or directly to the lipid bilayer of the rough endoplasmic reticulum, allowing transport across (Wiedmann *et al.*, 1987). A non-specific peptidase found on the luminal side of the endoplasmic reticulum, removes the signal peptide, and liberates the proα chains. This is a critical step, necessary for the formation of a functional procollagen molecule.

1.3.3 Intracellular Processing

Within the cisternae of the rough endoplasmic reticulum, the nascent chains are modified resulting in hydroxylation of prolyl and lysyl residues, and the glycosylation of hydroxylysyl residues (Figure 1.2). The catalytic reactions, brought about by three hydroxylases and two glycosyl transferases, occur both at the cotranslational and posttranslational level, until the triple-helix is formed which prevents further processing (Prockop *et al.*, 1976; Kivikikko & Myllylä, 1982).

1.3.3.1 Hydroxylation of prolyl and lysyl residues

All three hydroxylases require a non-helical peptide substrate with their susceptible prolyl and lysyl residues within a specific amino acid sequence (Kivirikko & Myllylä, 1985). For the hydroxylations the cosubstrates Fe^{2+}, ascorbate, molecular oxygen and 2-oxoglutarate, are also required.

Prolyl 4-hydroxylase catalyses the hydroxylation of prolyl residues in specific -X-Pro-Gly- sequences (Kivirikko, *et al.*, 1989). The 4-hydroxyprolyl residues in the chains

are essential for folding and thermal stabilisation of the newly synthesised triple helical molecule. As when inhibitors such as the iron chelator, α,α'-dipyridyl, are added *in vitro*, the triple-helices formed have a thermal denaturation temperature of 24 °C, as opposed to 40 °C for normally modified type I triple helices (Berg & Prockop, 1973). This makes prolyl 4-hydroxylase a target for pharmacological modulation, in the control of fibrotic disease (Kivirikko & Savolainen, 1988; Uitto *et al.*, 1984).

Prolyl 3-hydroxylase catalyses the hydroxylation of prolyl residues at the X position in specific -X-Y-Gly- sequences, and only when the Y is occupied by a 4-hydroxyprolyl residue (Tryggvason *et al.*, 1979). The function of 3-hydroxyproline is unknown, and its abundance varies in the different fibrillar collagens.

Lysyl hydroxylase (Turpeenniemi *et al.*, 1977) catalyses the hydroxylation of lysyl residues in the specific -X-Lys-Gly- sequence. It is tightly associated with the membrane of the rough endoplasmic reticulum, and reacts more efficiently with increasing lengths of peptide substrate. The hydroxyl groups have two functions: firstly, they act as the site of attachment for the carbohydrates, galactose and glycosyl-galactose, and secondly, they are crucial for the formation of intra- and inter-molecular cross-links which are essential in providing the tensile strength and mechanical stability of the collagen fibril.

1.3.3.2 Glycosylation of procollagen chains

The extent of glycosylation within the triple-helix is variable between different collagens, and in the same collagen in different tissues and at different ages (Royce & Barnes, 1977). The biological role of the covalently attached carbohydrate moieties remains unclear, but it is suggested that they have a role in the organisation of the collagen fibril. The inverse relationship between carbohydrate content and fibril diameter may be a result of a steric hindrance in the formation of highly ordered fibrils with the bulky sugars (Kivirikko & Myllylä, 1979).

Glycosylation occurs by the sequential action of two enzymes found, in the endoplasmic reticulum, hydroxylysyl galactosyl transferase and galactosyl hydroxylysyl glucosyl transferase, which transfer UDP-galactose to hydroxylysine and UDP-glucose to galactosyl-hydroxylysine residues respectively. Both enzymes require a non-helical conformation, a free ε-amino group in the substrate hydroxylysyl residue, and Mn^{2+}. The degree of glycosylation depends on the rate of triple-helix formation, and the longer the peptide the more effective the substrate. Other carbohydrate moieties are found in the non-helical C-propeptide of type I procollagen. Mannose-rich oligosaccharides are associated with asparagine residues in specific -Asn-X-Thr(Ser)- sequences in nascent proα chains located in the non-helical region (Clark, 1979). The function of these oligosaccharides is unknown.

1.3.3.3 Triple helix formation and disulphide bonding

The formation of the triple-helix is a complex folding process, which cannot take place until almost all the proline residues present in the Y position have been hydroxylated. It begins with the proper association of the three C-propeptides, and is followed by nucleation and propagation of the triple-helix in a C- to N- direction. The initial chain selection is thought to involve insertion of two proα1(I) and one proα2(I) chains into the endoplasmic reticulum at a common site, after attaching to a cell surface receptor/component via signal peptide sequences (Kirk *et al.*, 1987). Chain association and alignment is then initiated by non-covalent interactions between the C-terminal propeptides, which may include the interactions of conserved aromatic and hydrophilic residues (Brass *et al.*, 1992). Interchain disulphide bonds (catalysed by the protein disulphide isomerase) are then formed, stabilising the folded C-propeptides, which precedes triple-helix formation (Koivu & Myllylä 1987; Dölz & Engel, 1990). Folding of the triple-helix occurs by the nucleation of between three and ten -Gly-Pro-Hyp- triplets at the C-terminal of the collagenous domain. This is followed by a rapid propagation of the three chains folding together from the C- to N- terminal domain in a zipper-like mechanism, resulting in a fully folded procollagen molecule.

1.3.3.4 Secretion

Procollagen is transported from the rough endoplasmic reticulum into the Golgi complex, where it is packaged in the form of cylindrical aggregates in secretory granules, and then translocated into the extracellular space (Prockop *et al.*, 1976). The

rate of secretion is determined by the rate of synthesis of procollagen in the cisternae of the rough endoplasmic reticulum (Kao *et al.*, 1979).

1.3.4 Extracellular Processing

In the extracellular environment the N- and C- propeptides are cleaved from the procollagen molecule by two specific neutral zinc metalloproteinases, procollagen N-proteinase and procollagen C-proteinase, both of which require a triple-helical conformation and Ca^{2+} for activity (Davidson *et al.*, 1975).

1.3.4.1 Procollagen N-proteinase

Procollagen N-proteinase cleaves all three α chains at the N-terminal end of the major triple-helix liberating the N-propeptide and leaving a product of pC-collagen. Two enzymes have been identified, the procollagen type I N-proteinase, which excises the N-propeptides of types I and II procollagens, while the type III N-proteinase is specific to type III procollagen (Nusgens *et al.*, 1980).

Procollagen type I N-proteinase cleaves type I and II procollagen specifically at a -Pro-Gln- bond in the proα1(I) chain (Horlein *et al.*, 1978), and at a -Ala-Gln- bond in the proα2(I) and proα1(II) chains (Wozney *et al.*, 1981). The enzyme was first described in calf tissues (O'Hara *et. al.*, 1970), during the studies of the heritable connective tissue disease dermatosparaxis, characterised by severe skin fragility, found in cattle, and more recently in humans (Nusgens *et al.*, 1992). A lack of N-proteinase activity results in the accumulation of a fragment of procollagen containing the N-propeptide

(pN-collagen) and results in the formation of abnormal collagen polymers in the skin and other tissues (Lenaers *et al.*, 1971). Type I N-proteinase has since been detected in calf (Kohn *et al.*, 1974; Nusgens and Lapière, 1979; Colige *et al.*, 1995), chick embryo leg tendon (Leung *et al.*, 1979) and human skin (Steinmann *et al.*, 1980).

N-proteinase was first purified from chick tendons and whole chick embryos (Tuderman *et al.*, 1978; Tuderman & Prockop, 1982; Tanzawa *et al.*, 1985; Hojima *et al.*, 1989). The enzyme purified from chick embryo tendon (Hojima *et al.*, 1989) was a tetrameric complex of ~500 kDa, made up from four unreduced subunits of molecular mass 161, 135, 120 and 61 kDa, of which the two larger units had catalytic activity. A minor, possible degradative form, of 300 kDa, was also identified which retained catalytic activity. Recently an active 107 kDa procollagen N-proteinase was purified from extracts of foetal calf skin using concanavalin A-Sepharose and heparin-Sepharose chromatography (Colige *et al.*, 1995). It is suggested that the active N-proteinase purified in this study corresponds to a 84 kDa (unreduced) unit purified by Hojima *et al.*, (Hojima *et al.*, 1989). The 107 kDa N-proteinase also demonstrated specific binding to type XIV collagen, one of the FACIT (fibril-associated collagens with interrupted triple-helices) collagens. It has been proposed that type XIV collagen immobilises N-proteinase in the close vicinity of type I collagen fibres, allowing spatial control of the newly synthesised procollagen α chain processing and polymerisation (Colige *et al.*, 1995).

A 100 or 170 kDa type III N-proteinase was purified from calf tendon fibroblasts (Nusgens *et al.*, 1980), and more recently 72 kDa and 70 kDa type III N-proteinases

were purified from foetal calf aorta and human placenta, respectively (Halila & Peltonen, 1984; 1986). However, much less is known about type III N-proteinase, which removes the N-propeptides of type III procollagen by the cleavage of the -Pro-Gln- bonds.

1.3.4.2 Procollagen C-proteinase

Procollagen C-proteinase specifically removes the C-propeptide from pC-collagen and procollagen, and in doing so lowers the solubility approximately 10 000 fold. This step is critical in the initiation of the self assembly of collagen and pN-collagen into fibrils (Kadler *et al.*, 1987; Prockop & Hulmes, 1994). The enzyme specifically cleaves the -Ala-Asp- bond found between the junction of the C-telopeptide and C-propeptide regions in the α chains in type I and II procollagen (Dickson *et al.*, 1981; Fuller & Boedtker, 1981; Ninomiya *et al.*, 1984), and the -Gly-Asp- bond in type III procollagen (Kessler *et al.*, 1986). C-proteinase is a glycoprotein containing oligosaccharide side chains that binds to concanavalin A-Sepharose (Duksin *et al.*, 1978).

Over 20 years of research to characterise and determine the primary structure of C-proteinase, followed the first observations of C-propeptide removal, at neutral pH, in cultures of human and mouse fibroblasts (Goldberg *et al.*, 1975; Kessler & Goldberg, 1978). Despite significant efforts breakthroughs were a long time in coming, and at the start of this project little was known about the enzyme, as it is expressed at extremely low levels in connective tissue making purification very difficult. However,

this research has now culminated in the very recent discovery (three and a half years after beginning my project) that procollagen C-proteinase is identical to bone morphogenetic protein-1 (BMP-1) (Kessler *et al.*, 1996; Li *et al.*, 1996). BMP-1, is a member of the 'astacin'-zinc dependent endopeptidase family, and is capable of inducing newly formed bone when implanted into rodents at ectopic sites (Urist, 1965; Reddi & Huggins, 1972).

The history of procollagen C-proteinase research, the relationship between C-proteinase and BMP-1, and the importance of this discovery are discussed at length in sections 1.4 and 1.5.

1.3.5 Fibril Formation

Cleavage of the N- and C-propeptides in the extracellular space is imperative for the correct formation of collagen molecules, which then aggregate into long, unbranched, axially ordered fibrillar structures with a characteristic periodicity. From X-ray diffraction and electron microscopy of type I fibrils in tendon, a repeat period, D, of 67 nm is observed, and each individual collagen molecule is 4.4D in length (Miller, 1976). Further to this, each molecule in the fibril is staggered by 1D, a result of possible interactions between the hydrophobic and hydrophilic residues along the triple-helix (Hulmes *et al.*, 1973). These staggers create short spaces called the 'gap zones' which are thought to be required for cross-link formation, necessary for the stabilisation of the fibril.

A number of pathways for fibril assembly have been proposed from data obtained using *in vitro* fibril formation systems. Type I collagen can be extracted from connective tissues by solubilisation into ice cold acetic acid. Neutralisation and warming of acid soluble type I collagen results in the formation of fibrils (Wood, 1964). The self assembly of collagen molecules was shown to pass through lag, near-sigmoidal, and plateau growth phases when analysed turbidimetrically (Comper & Veis, 1977). From these studies two models of fibril formation were suggested. The first model begins with nucleation, where a critical number of collagen molecules interact, forming a stable nucleus, from which growth proceeds with monomer addition (Gelman & Piez, 1980; Na, 1989). The second model involves individual molecules forming small intermediate aggregates which assemble into larger fibrils (Trelstad *et al.*, 1976; Silver *et al.*, 1979; Ward *et al.*, 1986). It is, however, probable that certain aspects of both models occur in a complex process of fibrillogenesis. These models, however, were proposed on fibrils extracted under non-physiological conditions where the phosphate concentration was ≥30 mM, and the temperature was <35 °C.

Since then, the study of fibrillogenesis has been aided by the development of a cell-free *in vitro* system, which generates fibrils under physiological conditions by the sequential cleavage of the N- and then C- propeptides (Kadler *et al.*, 1987, 1988). Using this system, fibrils were shown to grow in a C to N terminal direction from a pointed tip. Growth is initially in a unidirection, but when monomers bind to the blunt end with a reverse polarity, the end result is a fibril with one blunt and one pointed tip (Holmes *et al.*, 1992).

Factors controlling and regulating fibril assembly, lateral growth accounting for varying fibril diameter, and general fibril morphology, are far from understood. However, the effects of both N- and C- propeptides on fibril diameter have been proposed during a number of studies. Thus the removal of the C-propeptide prior to the N-propeptide results in thin fibrils, whereas thick fibrils are formed if the N-propetide is removed first (Miyahara *et al.*, 1984). Recently, increased levels of C-proteinase at a constant concentration of pC-collagen has led to the formation of fibrils with reduced shape asymmetry, so the occurrence of bipolar fibrils is in part dependent on the concentration of C-proteinase (Kadler *et al.*, 1996).

1.3.6 Cross-linking

The formation of covalent inter- and intra- molecular cross-links, immediately follows the spontaneous aggregation of collagen molecules into fibrils (Eyre *et al.*, 1984; Light & Bailey, 1980). These cross-links are essential for the stabilisation of the fibrous forms of collagen, providing its tensile strength and mechanical properties. The enzyme lysyl oxidase, catalyses the oxidative deamination of the ε-amino group of specific lysyl and hydroxylysyl groups in the non-helical telopeptide regions (for review see Kagan, 1986). These aldehydes then spontaneously condense with other aldehydes or other ε-amino groups, forming a variety of di-, tri- and tetra- functional cross-links (Ricard-Blum & Ville, 1989). These cross-links can be formed between chains from the same type of collagen, or between different types of collagen, such as types I and III. Lysyl oxidase is dependent on a strict steric orientation of collagen, the sequence

of amino acids surrounding the target site, and requires a copper ion and an aromatic carbonyl as cofactors (Yamanchi & Mechanic, 1988).

Lysyl oxidase is first secreted into the extracellular space as a 50 kDa pro-enzyme, where it is converted to a 28 or 32 kDa functionally active enzyme (Trackman *et al.*, 1992). In recent studies the pro-enzyme secreted from fibrogenic cells in smooth muscle from arterial wall, has been shown to be converted into active lysyl oxidase by procollagen C-proteinase (Panchenko *et al.*, 1996). Procollagen C-proteinase therefore influences the rate and/ or extent of fibril formation in connective tissues at two important levels. Firstly, by cleaving the C-propeptide from soluble procollagen, and secondly by activating lysyl oxidase, which catalyses cross-links in the insoluble fibres.

When lysyl oxidase is inhibited, there is an accumulation of soluble collagen which is ultimately degraded preventing fibril formation (Vater *et al.*, 1979). It therefore follows, that inhibition of cross-link formation by acting on C-proteinase would suppress the overproduction of collagen in fibrotic disorders. This makes procollagen C-proteinase an exciting target for therapeutic inhibition, for use in many pathological situations.

1.4 TYPE I PROCOLLAGEN C-PROTEINASE

After the initial observations for C-proteinase activity in human and mouse fibroblasts, active enzyme has been observed in a number of connective tissues, including chick

calvaria (Fessler *et al.*, 1975; Davidson *et al.*, 1977), chick tendon fibroblasts (Uitto & Lichtenstein, 1976; Duksin *et al.*, 1978; Leung *et al.*, 1979), in extracts of chick calvaria (Njieha *et al.*, 1982), and chick embryo tendon (Hojima *et al.*, 1985). In addition to a specific C-peptidase, two cathepsin like proteases were shown to cleave C-propeptides from type I procollagen at acid pH. A cathepsin D like enzyme, isolated from chick embryo tendon and cultured tendon fibroblasts (Davidson *et al.*, 1979), and cathepsin D, isolated from adult chicken livers (Helseth & Veis, 1984) both specifically cleaved the C-propeptide at a protease-susceptible site situated close to the authentic C-proteinase cleavage site.

The authors put forward a model for intracellular processing of procollagen in secretory granules initiated by cathepsin D, which normally acts as a lysosomotrophic agent in the degradation of proteins. However, the presence of a specific intracellular cathepsin like enzyme responsible for initiation of fibril formation, by removal of the C-propeptide was disproved by the purification of a metalloproteinase which cleaved at a specific -Ala-Asp- site that marks the junction between the C-telopeptides and the C-propeptides at neutral pH (Hojima *et al.*, 1985). Site directed substitution of the conserved Asp in the proα2(I) chain completely blocked chick C-proteinase cleavage confirming the catalytic site for C-proteinase was the -Ala-Asp- (Lee *et al.*, 1990).

The 97-110 kDa C-proteinase was purified to apparent homogeneity from organ explant culture media from 390 dozen chick embryos, using three affinity columns (Green A DyeMatrex, concanavalin A-Sepharose and heparin-Sepharose), and two gel filtration columns (Sephacryl S-300 and S-200) (Hojima *et al.*, 1985). Two forms of

C-proteinase were separated by heparin-Sepharose chromatography which cleave the C-propeptides from type I procollagen with equal efficiency (Hojima *et al.*, 1985). The forms, termed E-A and E-B, have molecular weights of 110 and 95 kDa respectively, and are present in a ratio of 2:1 (E-A:E-B). This ratio appears to be reversed during long incubations of the chick embryo leg tendons during culturing. In the *in vitro* fibril assembly system only the E-A form of C-proteinase will cleave the C-propeptides from pC-collagen, and generate fibrils (Kadler *et al.*, 1987). From these two observations E-B may be a processed form of E-A, a partially degraded form of E-A, or it may have a different level of glycosylation compared to E-A.

1.4.1 Characteristics of Procollagen C-proteinase

The pH optimum of C-proteinase activity on procollagen is between 8.0 and 8.5. Activity is reduced to 80% at pH 7.5, and below pH 6.0 all activity is lost. The stability of C-proteinase is also dependent on the pH being in a range of 6-9, compared to pH 4.3 where a 20% loss in activity is registered (Hojima *et al.*, 1985). The presence of calcium, at an optimum concentration of 5-10 mM, and the absence of other divalent ions, such as Zn^{2+}, Mg^{2+}, and Cd^{2+}, is imperative for activity and enzyme stabilisation at physiological temperatures. The K_m and the κ_{cat} values of 96 nM or 75 nM and $41h^{-1}$ or $32h^{-1}$, were determined using the direct linear plot (Eisenthal & Cornish-Bowden, 1974) and the Lineweaver-Burk plot, respectively. The activation energy, determined from an Arrhenius plot, was 21 000 cal mol^{-1}.

1.4.1.1 Inhibitors of C-proteinase

Inhibitors of type I procollagen C-proteinase are shown in Table 1.2. The extent of inhibition is expressed as a percentage of cleavage relative to controls without inhibitor added. A number of divalent metal ion chelators, such as EDTA and EGTA, irreversibly inhibited C-proteinase activity at a range of concentrations (Hojima *et al.*, 1985). The most potent inhibitor to C-proteinase activity was *o*-phenanthroline, inhibiting activity by 49% at 0.1 mM. SDS, FCS and zinc chloride all inhibited up to 100% at low concentrations. Reducing agents β-mercaptoethanol and phosphates, and basic amino acids lysine and arginine were partially active between 1-10 mM and 5 mM respectively. Specific serine protease inhibitors, soybean inhibitor and PMSF, were not effective on C-proteinase activity at 100 μg/ml and 5 mM concentrations respectively. Also, the specific cysteine protease inhibitors, N-ethylmaleimide and E-64 were ineffective at 5 mM concentrations. The lack of inhibition from these specific protease class inhibitors, confirms that the purified enzyme is a member of the zinc metalloprotease family.

Tunicamycin, an inhibitor of the lipid-carrier dependent protein glycosylation, causes an accumulation of pC-collagen and impaired conversion to collagen in cell cultures, by acting either on the activation, secretion, stability or activity of C-proteinase (Duksin and Bornstein, 1978; Duksin *et al.*, 1978). Other evidence for the presence

Table 1-2 Inhibitors of Type I Procollagen C-proteinase

Inhibitor	Concentration	% Inhibition of activity
o-Phenanthroline	0.1 mM 1 mM	49 95
EDTA	5 mM	100
EGTA	5 mM	100
α,α'-Dipyridyl	5 mM	100
β-Mercaptoethanol	1 mM	76
SDS	50 μg/ml	100
Foetal calf serum	5%	100
Zinc chloride	0.1 mM	97
Sodium phosphate	10 mM	91
Concanavalin A	50 μg/ml	77
Pepstatin A	0.4 mM	80
Lysine	5 mM	68
Arginine	5 mM	45
E-64	5 mM	0
N-Ethylmaleimide	5 mM	6
Soybean inhibitor	100μg/ml	0
PMSF	2 mM	20

Data source from Hojima *et al.*, 1985

of carbohydrate includes the inhibition of C-proteinase activity by the lectin concanavalin A which targets these moieties, and at 50μg/ml inhibits activity by 77% (Hojima *et al.*, 1985). Synthetic peptides at relatively high concentrations, with sequence analogy to cleavage sites in type I procollagen, were inhibitory to C-proteinase and not N-proteinase. The peptide Tyr-Tyr-Arg-Ala-Asp-Asp-Ala inhibited cleavage by 45 and 60% at 6 and 12 mM respectively, but the shorter peptides such as Ala-Asp-Asp-Ala were less effective, with 30% inhibition at 26 mM (Njieha *et al*, 1982).

1.4.2 Procollagen C-proteinase Enhancer molecule

A 125 kDa type I procollagen C-proteinase was first purified from the medium of cultured mouse fibroblasts using ammonium sulphate precipitation, gel filtration, and affinity chromatography on a column of Sepharose coupled to the C-propeptide of type I procollagen (PP-Sepharose) (Kessler *et al.*, 1986). The properties of the C-proteinase were similar to those shown by the 97-110 kDa chick C-proteinase (Hojima *et al.*, 1985). They were both found in an extracellular source, acted at neutral or slightly higher pH (8.5), were dependent on zinc for activity, and cleaved the C-propeptides at the -Ala-Asp- bond. However, major losses of C-proteinase activity were encountered during purification, with only a 4.7% recovery of activity over the three stages.

It was subsequently shown that when contaminating proteins were removed using a lysyl-Sepharose column, a near homogeneous 80 kDa active C-proteinase was

separated from a 55 kDa glycoprotein (Kessler & Adar, 1989). Activity of this purified C-proteinase was enhanced 7 fold when the enzyme was reconstituted with saturating amounts of the 55 kDa protein, and the smaller 36 and 34 kDa proteolytically processed forms. It was concluded that full expression of mouse C-proteinase activity was dependent on these so called enhancer molecules (Adar *et al.*, 1986; Kessler & Adar, 1989). The three forms of the enhancer molecule are immunologically related, show no intrinsic procollagen processing ability, and are all capable of enhancing C-proteinase activity.

From proteolytic fragments of the 55 kDa enhancer protein, amino acid sequences were obtained which matched to a cDNA clone, with unknown function, derived from a mouse astrocyte library (Takahara *et al.*, 1994a). This mouse cDNA clone was amplified and used as a probe to isolate the human enhancer cDNA from a human placenta library. The molecular weight of the mouse and human mature forms were determined as 50 and 45 kDa respectively, with the mouse exhibiting greater posttranslational modifications.

From immunoblotting the enhancer proteins are most abundant in connective tissues, particularly in tendon, which is rich in collagen type I, suggesting there is correlation between enhancer expression and the deposition of collagen (Kessler *et al.*, 1990; Takahara *et al.*, 1994a). Type I collagen and the C-proteinase enhancer may be also be coregulated as both the PCOLCE, the gene encoding for the human enhancer protein, and the COL1A2, gene encoding for proα2(I), are mapped to the same location on human chromosome 7 (Takahara *et al.*, 1994a).

The 36 and 34 kDa forms of the enhancer are derived from the amino-terminal portion of the 55 kDa form. This region of the 55 kDa protein contains two copies of a CUB (Complement-Uegf-BMP-1) domain, which is found in proteins involved in embryogenesis and organogenesis (Bork & Beckmann, 1993). As the 36 kDa form contains only these CUB domains, it is suggested that these are involved in enhancer-propeptide binding, which induces a procollagen conformational change, rendering the -Ala-Asp- cleavage site more accessible to C-proteinase. CUB domains are also found in members of the zinc dependent astacin family such as human BMP-1 (Wozney *et al.*, 1988) and *Drosophila* Tolloid (DrTld) (Shimell *et al.*, 1991). These proteases are discussed in the following section. It was this significant observation and suggestion of a possible association with C-proteinase, that led to the discovery that C-proteinase was identical to BMP-1.

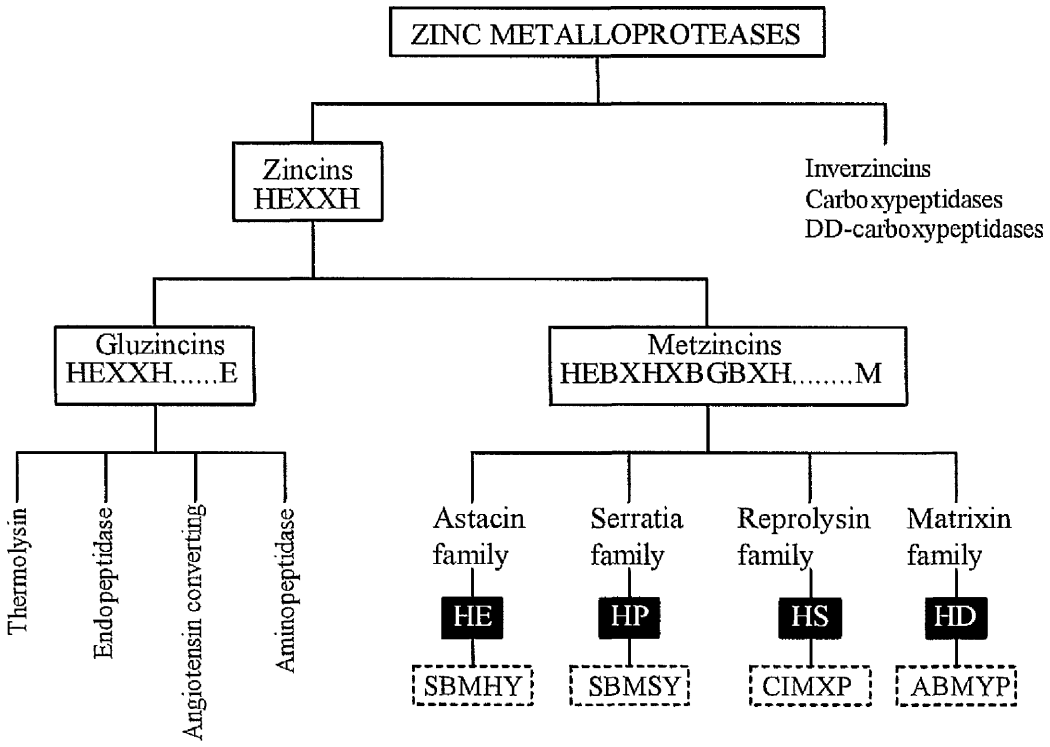
1.5 METZINCINS

Metalloproteases are involved in a number of diverse processes including the degradation of the ECM, embryonic development, bone formation, cancer and arthritis. Over the past few years there has been a dramatic increase in numbers of identified zinc metalloproteases, which has prompted a new classification scheme where the proteases are split into four superfamilies: zincins, inverzincins, carboxypeptidases and DD-carboxypeptidases (Hooper, 1994). A schematic of the relationship between members of the zinc metalloprotease family is shown in Figure 1.3. Members of the zincin superfamily possess the HEXXH zinc binding motif (Bode *et al.*, 1993) and are further divided into the gluzincin and metzincin families. The gluzincins have a short

HEXXH consensus sequence containing the zinc ligands and glutamic acid acting as the third ligand, whereas, the metzincins contain a longer HEBXHXBGBXH consensus sequence. The metzincins are divided into four families (astacin, serratia, reprolysin, and matrixin) which are distinguished firstly, by the residue following the third histidine zinc ligand, and secondly by, the residues surrounding a methionine-containing turn (Met-turn) (see Figure 1.3).

The serratia family includes the plant pathogen bacterial proteases from *Serratia* sp. and proteases B and C from *Erwinia chrysanthemi* (Nakahama et al., 1986). Members of the reprolysin family include snake venom proteases, and non- and haemorrhagic toxin proteins. The matrixins are the matrix degrading metalloproteinases (MMPs) including collagenases, stromelysins and gelatinases (for reviews see Matrisian, 1992; Birkedal-Hansen *et al.*, 1993). They are critical for normal matrix turnover, including bone growth and remodelling, however, when the co-ordinated regulation of MMPs and their natural inhibitors TIMPs (Murphy & Docherty, 1992) is disrupted, a number of degradative diseases ensue (Murphy *et al.*, 1991). MMPs play an important role in collagen turnover as they degrade old and damaged tissue, such as collagenase in chronic wounds which clears debris, before new matrix can be synthesised and laid down. The matrixins are therefore major target sites for the pharmacological modulation of diseases such as arthritis, cancer and peridontitis.

Figure 1.3 Schematic of Zinc Metalloprotease Families



H represents Histidine; B represents any bulky, apolar residue; X represents any amino acid. Individual families of the metzincins are distinguished by (1) the residues following the third zinc ligand which are indicated in the black boxes, and (2) the residues surrounding the Met, which are shown in the hatched lined boxes.

1.5.1 Astacin family

Over 17 zinc proteases are members of the astacin family which all possess the characteristic 18 amino acid consensus sequence HEXXHXXGFXHEXXRXDR (for review see Bond & Beynon, 1995). The family was named after the smallest member astacin, a digestive enzyme from the crayfish *Astacus astacus* (Dumermuth *et al.*, 1991) which was the first to be biochemically characterised and sequenced (Titani *et al.*, 1987). Members of the family are expressed in a tissue specific manner in mature systems and are temporally and spatially expressed in developmental systems. Major activities of proteins are seen in hatching, and dorsal/ventral patterning in a number of diverse organisms (Table 1.3).

1.5.1.1 Domain structures

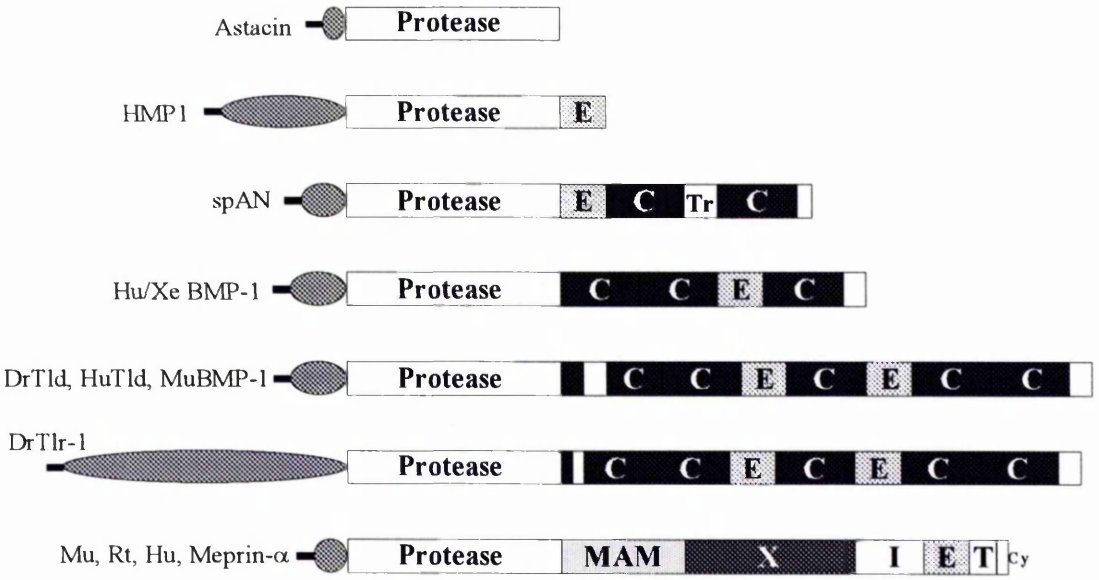
All the members of the astacin family contain a consensus sequence, with the HEXXH zinc binding motif, within a 200 amino acid protease domain. A schematic of the domain structures of members of the astacin family are shown in Figure 1.4. All members, apart from Quail CAM-1 (QuCAM-1), have a signal peptide, responsible for directing the protein into the endoplasmic reticulum, and prosequences which vary in length from ~50 to 520 amino acids. The largest prosequence found in Dr Tlr-1 is thought to prevent expression of protease activity in the early stages of embryogenesis, when cell cycles are prolonged (Nguyen *et al.*, 1994). The larger members also contain a number of non-catalytic C-terminal domains which are thought to be

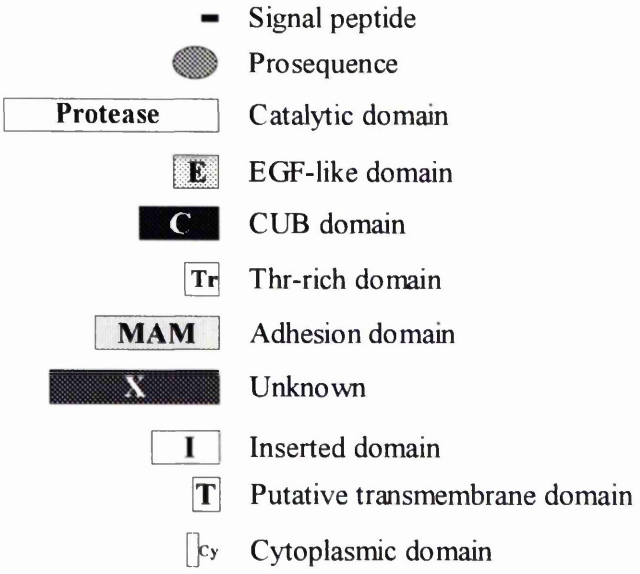
Table 1-3 Distribution and Functions of Astacin Family Members

Member	Species	Proposed function	References
Astacin	Crayfish stomach	Digestion/hepatic/pancreatic	Vogt *et al.*, 1989
CAM-1	Quail embryos	Hatching induced by degradation of eggshell	Elaroussi & DeLuca, 1994
HMP-1	Hydra tentacles	Morphogenesis of tentacle cells and pattern formation	Yan *et al.*, 1995
spAN and BP10	Early stages in sea urchin embryogenesis	Differentiation of ectodermal linkages and subsequent patterning	Lepage *et al.*, 1992; Reynolds *et al.*, 1992
BMP-1	Human and mouse embryos	Bone/ cartilage formation, biominerilisation and pattern formation	Wozney *et al.*, 1988
Tolloid (TLD)	*Drosophila*, mouse and human embryos	Early dorsal/ventral patterning	Ferguson & Anderson, 1992
Tolloid-related-1 (Dr Tlr-1)	*Drosophila* embryo	Essential in larval and pupal development	Nguyen *et al.*, 1994
Meprin A	Mouse kidney, rat and human intestine, embryos	Degradation of peptides such as parathyroid hormone and ECM	Wolz & Bond, 1995; Kaushal *et al.*, 1994

Information is composed from Bond & Beynon, 1995.

Figure 1.4 Domain Structures of Members of the Astacin Family





All sequences are derived from cDNA. Species are indicated as: Hu, human; Xe, *Xenopus*; Dr, *Drosophila*; Mu, mouse; Rt, rat.

involved in protein-protein substrate interactions. These include a varying number of EGF-like motifs and/or CUB domains. The CUB domain is referred to as such, as the modules are found in the complement subcomponents C1r/C1s (Tosi *et al.*, 1987), embryonic sea urchin Uegf (Delgadillo-Rosso *et al.*, 1989), and BMP-1 (Wozney *et al.*, 1988).

The EGF-like domain consists of 40 amino acids and contains six highly conserved cysteine residues linked by interdomain disulphide bonds. The domains have been found in a number of different proteins including coagulation factors X and IX and fibroblast proteoglycan (Krusius *et al.*, 1987). However, the function remains unclear, with suggestions that they may be involved in calcium binding as well as protein-protein interactions. CUB domains consist of approximately 100 amino acids, with four highly conserved cysteine residues which probably form disulphide bonds, as seen in C1r/C1s. There are several conserved blocks interrupted by regions of variable length, and conserved regions of hydrophobicity indicate that a β-parallel sheet structure is formed, similar to that seen in the immunoglobulins (Bazan, 1990). However, even with a relatively conserved structure, different binding specificity and functions are observed, probably a result of evolution assembling proteins as modules, ensuring different specific proteases were created (Patthy, 1985).

1.5.2 Bone Morphogenetic Protein-1

Extracts of demineralised bone which initiate *de novo* cartilage and bone formation, when implanted into ectopic sites in rodents, were first identified by Urist and then

Reddi & Huggins as bone morphogenetic proteins (BMP) (Urist, 1965; Reddi & Huggins, 1972). BMP is the active component, which, at the implanted site initiates the differentiation of migrating mesenchyme into chondroblasts and chondrocytes, and then subsequent replacement with osteoblasts and osteocytes (Reddi, 1981). A number of other factors, such as Fibroblast Growth Factor basic (FGFb) and Transforming Growth Factor β1 (TGFβ1) which have variable effects on bone formation and regulation, may also contribute to the activities of BMP (Baylink *et al.*, 1993).

BMP-1 was originally copurified with three TGFβ-related molecules, BMPs 2A, 3 and 4 from demineralised bone extract (Wozney *et al.*, 1988). Subsequently three more BMPs (BMP 5, 6 and 7) have been identified and were shown to be part of the TGFβ superfamily (for review see Kingsley, 1994), based on primary amino acid sequence homology including the highly conserved seven cysteine residues (Wozney, 1992). BMP-1, however, through isolation of the human cDNA clone, was shown to have structural similarity to members of the zinc dependent astacin family.

The domain structures of the human and *Xenopus* BMP-1, and human and *Drosophila* Tld and Tlr-1 are very similar, except that the Dr Tld and Dr Tlr-1 generally have two extra CUB domains and one extra EGF-like domain (Figure 1.4). The mouse BMP-1 and Tld are the only proteins with identical domain structures (Fukagawa *et al.*, 1994). Evidence has also emerged that the BMP-1 gene produces alternatively spliced transcripts, which encode for shorter BMP-1 variants and a protein which is longer but

structurally similar to Tld and is designated mammalian TLD (mTld) (Takahara *et al.*, 1994b).

Also further evidence confirming the relationship between Dr Tld and BMP-1 involves the relationship with the TGFβ-like proteins. Firstly, as BMP-1 was copurified with BMPs 2-4, it has been suggested that BMP-1 may actually be acting on these TGFβ-like proteins. Secondly, it has been shown that null mutations in Dr Tld leads to partial transformation of the dorsal ectoderm into ventral ectoderm (Irish & Gelbart, 1987; Padgett *et al.*, 1987), a phenotype, not as severe, but similar to that caused by a mutation in decapentaplegic (dpp) which itself has homology with BMP 2 and 4 (Padgett *et al.*, 1993). From structural evidence, that Dr Tld and dpp act antagonistically (Childs and O'Conner, 1994; Ferguson & Anderson, 1992), and that BMP-1 probably acts on TGFβ (Kessler *et al.*, 1996), it is suggested that these gene products are probably part of the same highly conserved complex signalling/protease cascade. Where it is proposed that BMP-1/Tld- like proteins associate with unprocessed TGFβ-related molecules, and via the protease domain cleave and releases biologically active C-terminal regions (Fukagawa *et al.*, 1994).

1.5.2.1 Identification of BMP-1 being identical to procollagen C-proteinase

A major breakthrough in the field of BMP-1 and procollagen C-proteinase research came at the end of my three and a half years research, in January 1996, when two independent groups showed that they are identical proteins (Kessler *et al.*, 1996, Li *et al.*, 1996). Firstly, Kessler *et al.*, assumed that C-proteinase would probably have a

CUB domain for protein-protein interactions with the enhancer molecule, which binds to the C-propeptides by means of such a domain (Takahara, *et al.*, 1994a; Section 1.4.2). Recombinant BMP-1 was then produced by a baculovirus system, and shown to cleave type I procollagen yielding pNα1(I) and pNα2(I) chains. Furthermore, BMP-1 cleaved type I procollagen specifically yielding the products that were indistinguishable from those cleaved by the mouse C-proteinase purified by Kessler & Adar, (Kessler & Adar, 1989). Cleavage of procollagen by BMP-1, was enhanced 7 fold by the enhancer molecule, and BMP-1 specific antibodies recognised the mouse C-proteinase. The final evidence was that the amino acid sequences of mouse C-proteinase fragments, produced by V8 treatment, aligned with the published sequence of BMP-1 (Wozney *et al.*, 1988; Takahara *et al.*, 1994b; Fukagawa *et al.*, 1994).

Secondly, Li *et al.*, adopted a more direct approach using amino acid sequences from tryptic peptides of chick C-proteinase, separated by a reverse phase C18 column, to design primers for reverse transcription-PCR (Li *et al.*, 1996). The PCR product was 592 bp, similar to that predicted from the amino acid sequence of BMP-1 (Wozney *et al.*, 1988). Five positive cDNA clones from a human skin fibroblast library, whose sequences encoded all the amino acids in the chick C-proteinase tryptic peptides were obtained. Analysis of these clones, and overlapping sequences indicated two proteins of 730 and 986 amino acids. The longer clone had the same sequence as the longer mTld of alternatively spliced BMP-1 (Takahara *et al.*, 1994b). The cDNA clones were then expressed in the human tumour cell line HT1080, and medium from transfected cells, from both cDNAs, were shown to cleave type I procollagen into pNα1(I), pNα2(I) chains, and a disulphide linked C-propeptide trimer.

As BMP-1 is also linked to Dr Tld-like proteins, involved in regulation of pattern formation in embryogenesis, it brings together a new concept that the BMP-1/ C-proteinase has, at least, dual functions as it is also critical and involved in matrix deposition. The effects of BMP-1 on bone morphogenesis probably cannot be explained by its C-proteinase activity, which suggests that alternative splicing of BMP-1, as already observed (Takahara *et al.*, 1994b), is probably responsible for the generation of a number of C-proteinases able to cleave different procollagens and TGFβ-like proteins (Bond & Beynon, 1995). This opens up the field into BMP-1/ C-proteinase research and represents the starting point for some exciting discoveries. Determining the enzymatic mode of action of the enzyme will lead firstly to a better understanding of fibrosis, characterised by an excessive deposition of collagen, and secondly whether C-proteinase can be pharmacologically modulated by inhibition.

1.6 FIBROTIC DISORDERS

Fibrotic disorders of organs and tissues, such as liver, lung and heart that result in the destruction of tissue architecture and function, are major causes of human morbidity and mortality. Additionally in wound healing, hypertrophic dermal scarring and keloid scars can result in disfigurement and disability (Rockwell *et al.*, 1989), and scarring of the central nervous system, following traumatic injury, can result in impaired neural regeneration. The progressive fibroproliferative diseases such as liver cirrhosis (Mezey, 1991), pulmonary fibrosis (Heppleston, 1991), scleroderma (Mauch *et al.*, 1993), atherosclerosis and rheumatoid arthritis (Harris, 1990) are characterised by the excessive ectopic production and disorganised deposition of connective tissue.

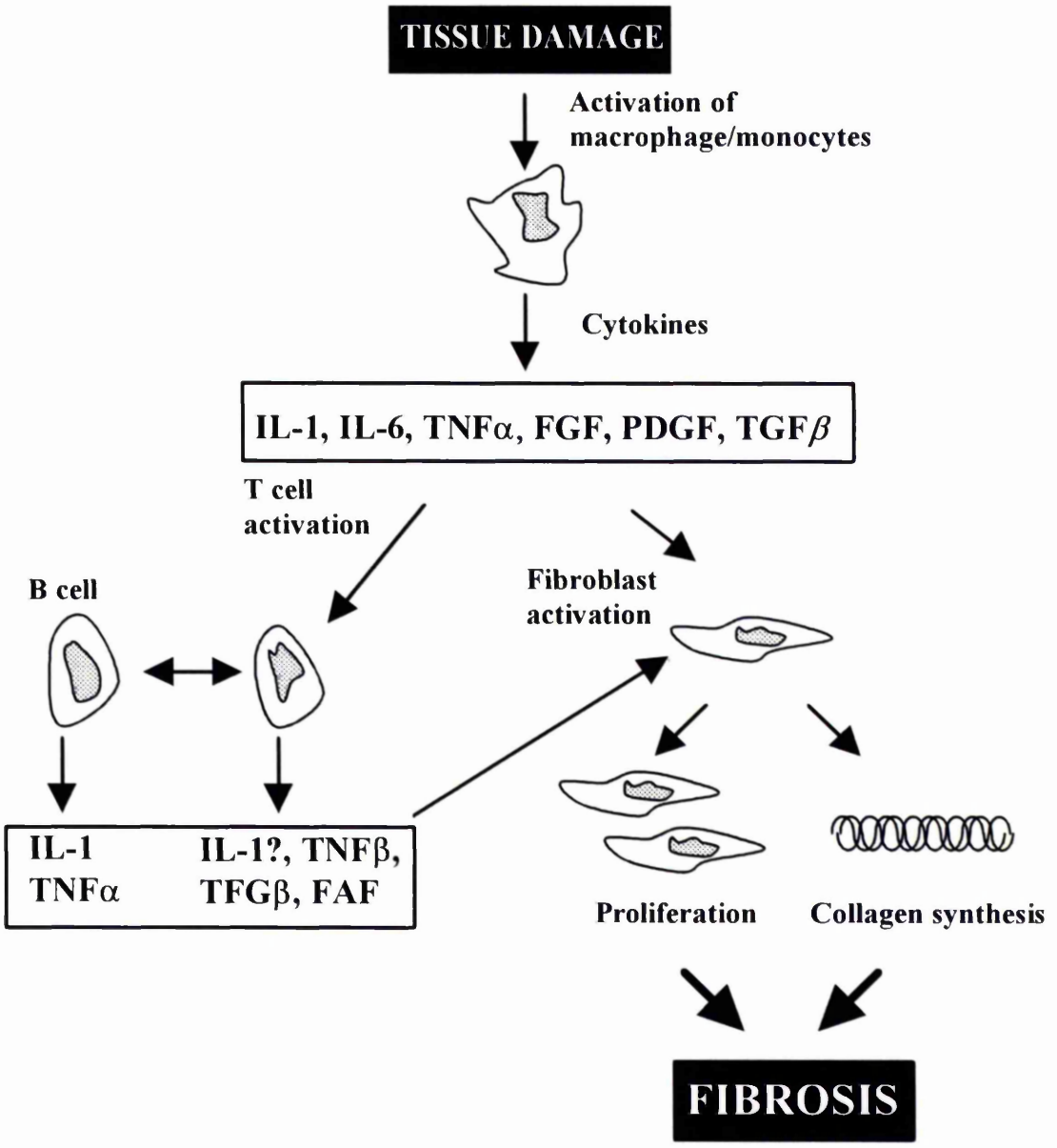
Current treatments for fibrotic disease are limited and generally have little effect upon the pathological progression of fibrogenesis, and novel therapies and strategies are sought (Franklin, 1995).

All forms of fibrosis have similar cellular and humoral events which lead from the primary tissue damage to the eventual deposition of connective tissue and ECM (Sporn & Harris, 1981) (Figure 1.5). These events targeted in therapeutic strategies fall into three groups: the direct attack on growth and proliferation of connective tissue using cytotoxic drugs, the modulation of cytokines which regulate proliferation and biosynthetic activities, and modulation at the direct control of collagen synthesis and secretion (Franklin *et al.*, 1991).

1.6.1 Direct Attack and Anti-Inflammatory Therapy

Cytotoxic drugs have been used to slow the proliferation of fibroblasts in pulmonary fibrosis with limited success (Casas *et al.*, 1987). Another therapeutic strategy is to treat the chronic inflammatory response, when tissue damage persists, prior to fibrogenesis. The treatment with steroids has shown some promise in liver and pulmonary fibrosis (Friedman, 1993; Crystal *et al.*, 1984), and has reduced the severity of hypertrophic scarring (Rockwell *et al.*, 1986). However, the side effects of this systemic therapy are unacceptable and limit its use (Haynes and Maud, 1985). Non-

Figure 1.5 Schematic of Cellular and Humoral Events Leading to Fibrosis



steroidal anti-inflammatory drugs developed to eradicate the side-effects of steroids has shown no worthwhile benefits in chronic disease.

1.6.2 Modulation of Cytokines

Activated macrophages produce at least six growth factors interleukin-1 (IL-1), tumor necrosis factor α (TNFα), interleukin-6 (IL-6), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and TGFβ1, which have all been shown to effect fibroblast proliferation and metabolism (Kovacs, 1991). TFGβ and PGDF are both highly fibrogenic. PGDF is the major mitogen for fibroblasts. Whereas, TGFβ1 is the most potent agonist for the fibrogenic mechanism, by increasing the production of ECM (Sporn *et al.*, 1986; Brenner *et al.*, 1994), and reducing the degradation of the ECM by inhibiting MMPs and reciprocally increasing TIMP mRNAs (Matrisian, 1990). TFGβ, produced and secreted by inflammatory cells and platelets, as discussed before, also has proposed roles in embryonic development, as well as tumorigenesis, immunosuppression, and wound healing (Sporn & Roberts, 1988; Rizzino, 1988).

The use of TGFβ1 as a therapeutic target in the modulation of fibrogenesis has produced varying degrees of success. TFGβ1 antibodies have reduced scarring in rat dermal injury (Shah *et al.*, 1992), and depressed ECM deposition in experimental glomerulonephritis (Border *et al.*, 1990). However, it would not be feasible to administer these antibodies for the prolonged duration of the disease unless they were 'humanised' to reduce the immunogenecity. Different approaches are now being

sought, looking into interactions with TGFβ1 receptors, and the possible inhibition of the signal transduction mechanism (Franklin, 1995).

1.6.3 Direct Inhibition of Collagen Synthesis

As the predominant overproduced components in fibrotic connective tissue are type I and III collagen, attempts to control these, have been aimed at the posttranslational level of collagen synthesis. Two key enzymes, crucial to collagen synthesis, prolyl 4-hydroxylase and lysyl oxidase have been targeted, with promising results.

1.6.3.1 Inhibition of prolyl 4-hydroxylase

Activity of prolyl 4-hydroxylase is essential for the formation of triple helices and subsequent secretion into the ECM (Kivirikko *et al.*, 1989). Most drug approaches, so far, have used analogues to α,α'-dipyridyl, an iron chelator, and to 2-oxoglutarate, an essential cofactor. The use of α,α'-dipyridyl has been ruled out due to the undesirable side-effects owing to iron depletion (Casas *et al.*, 1987). Competitors to 2-oxoglutarate, pyridyl 2,4- and 2,5- dicarboxylic acids, effectively inhibit the enzyme (Franklin *et al.*, 1991). However, at physiological pH, the C-groups are ionised and the molecules prevented from crossing the plasma membrane into the intracellular environment of the endoplasmic reticulum (Tschank *et al.*, 1987). Ester and amide derivatives of these molecules, designed to diffuse more freely across the cytoplasmic membrane, have shown inhibition *in vitro*, but, so far, have shown no evidence of *in vivo* potency (Tschank *et al.*, 1991). HOE 077 a prodrug of 2,4-pyridyl-dicarboxylic acid has reached phase II clinical development for the treatment of liver fibrosis, but its

in vivo activity is not understood (Clement *et al.*, 1991). Current research includes a different approach, using derivatives of N-oxalyglycine, an analogous compound to 2-oxoglutarate (Karvonen *et al.*, 1990, Franklin *et al.*, 1991).

1.6.3.2 Inhibition of lysyl-oxidase

As discussed earlier (Section 1.3.6) lysyl oxidase is responsible for catalytic events leading to inter- and intra- chain cross-links stabilising collagen (Kagan, 1986). The naturally occurring β-aminoproprionitrile and various analogues have all been shown to depress collagen accumulation (Uitto *et al.*, 1984). However, their use as clinical agents is limited, due to non-specificity and toxicity. Using information from the cDNA clone for lysyl-oxidase it has been suggested that antisense oligonucleotides could be used to block translation of its mRNA (Trackman *et al.*, 1990).

1.6.4 The Way Forward

Current approaches to control fibrosis have mainly been at the post-translational level with limited success, with the main obstacle being the inability of compound diffusion through the cytoplasmic membrane. Procollagen C-proteinase is fundamental and necessary for collagen biosynthesis at two levels:

- Firstly, it reduces the solubility of the soluble precursor, procollagen, approximately 10 000 fold, by removing the C-propeptide, which is critical for the subsequent initiation of the self assembly of collagen into fibrils, in a structure essential to collagen lysine oxidation (Prockop & Hulmes, 1994).

- Secondly, it converts latent prolysyl oxidase into a functional catalyst which oxidises lysine in the newly assembled fibrils, forming stabilising cross-links (Panchenko *et al.*, 1996).

I suggest that, if procollagen C-proteinase was specifically inhibited, a reduction in the formation of collagen would result from depressing the above two functions. An inhibition at two distinct regulatory levels, which forms a new therapeutic approach to control fibrosis. This would eventually lead to the exciting development of inhibitors for the pharmacological modulation of all fibrotic disorders.

1.7 OUTLINE OF PROJECT

The first priority was to repeat and optimise the purification of type I C-proteinase from chick embryo tendon, developed by Hojima *et al.*, (Hojima *et al.*, 1985). The rate of chick dissection in the laboratory, prior to the start of my project, was 25 doz a week, which was prolonging the accumulation of starting material, where 4-5 L of organ culture media was required per purification. To increase this, the rate of dissection and resultant culturing would have to be scaled up, to obtain enough chick C-proteinase to obtain an amino acid sequence.

The optimised purification protocol would then be used to purify C-proteinase from a novel human source. Human cancer cell lines would be examined, as often, the condition is associated with excessive overproduction of collagen and hence C-proteinase activity. Cytokine profiling, on any cell line which could be used to obtain human C-proteinase, would be carried out, to determine what growth factors could be

added to media to either substantially increase levels of C-proteinase, or regulate C-proteinase. Hopefully enough human C-proteinase could be then be purified, to homogeneity, to obtain an amino acid sequence.

The main thrust of the project is to obtain a cDNA clone from purified C-proteinase. N-terminal amino acid sequencing of purified C-proteinase would allow the production of oligonucleotides for screening cDNA libraries. Alternatively, antibodies raised to purified enzyme would be used to screen human libraries. The cDNA clone would be used in a number of ways:

- To determine the primary structure
- To examine the biosynthesis of the enzyme
- For use in *in situ* hybridisations, in fibrotic tissue samples, to identify the location and activity of C-proteinase
- To examine interactions with the numerous growth factors, such as TGFβ1, involved in enhancing collagen synthesis
- To use purified enzyme in assays to evaluate the potency of compounds, which are being developed at the University of Manchester and British Biotech

2. MATERIALS AND METHODS

2.1 GENERAL METHODOLOGY

The highest quality of chemicals was used throughout, and all solutions were prepared using ultrapure water obtained from a Millipore reverse osmosis and ultrapure apparatus. The pH of solutions was measured using the Radiometer pHM82 model with a glass electrode at room temperature, and pH was corrected using either HCl or NaOH. A list of suppliers is given in appendix 1.

2.2 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS AND FLUOROGRAPHY

2.2.1 SDS Polyacrylamide Gel Electrophoresis

Protein samples were analysed by discontinuous polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) in the presence of SDS (SDS-PAGE), in an Anachem V2 20 x 20 cm slab gel system, consisting of a 7-15% (w/v) polyacrylamide separating gel, overlaid by a 3.5% (w/v) polyacrylamide stacking gel (Appendix 2 for details of gel solutions). Proteins were prepared for analysis by adding 0.25 volumes of 5 x sample buffer, consisting of 0.625 M Tris-HCl buffer, pH 6.8, containing 50% (v/v) glycerol, 10% (w/v) SDS and 0.005% (w/v) bromophenol blue. The samples were then reduced by heating at 100 °C for 3 min in the presence of 2% (v/v) 2-mercaptoethanol. After electrophoresis, gels were either stained with Coomassie Blue (Section 2.2.2.1), stained with ammoniacal silver nitrate solutions (Section 2.2.2.2), prepared for fluorography (Section 2.2.3), or transferred to polyvinylidene difluoride (PVDF) membrane (Section 2.2.4).

2.2.2 Protein Staining

2.2.2.1 Coomassie blue staining

Coomassie Blue was used to stain SDS-PAGE gels to visualise proteins. Proteins were stained for 1 hr at room temperature in 200 ml of 0.1% (w/v) Coomassie Blue in 30% (v/v) methanol and 10% (v/v) glacial acetic acid. The stain solution was removed and replaced with 200 ml of 30% (v/v) methanol and 10% (v/v) glacial acetic acid (destain) for 1 hr with repeated changes.

2.2.2.2 Silver staining

The gel was soaked in 50% (v/v) methanol for 2 x 30 min and then washed in water for 2 x 10 min. Proteins were stained with 300 ml of 0.8% (w/v) silver nitrate solution, containing 0.083% (v/v) sodium hydroxide and 0.006% (v/v) ammonia solution for 15 min. The gel was then washed in water for 1 hr with frequent changes, and then 500 ml of 0.005% (w/v) citric acid and 0.55% (v/v) formaldehyde added until protein bands were visible. After developing and washing in water, the gel was stored in 5% (v/v) glacial acetic acid.

2.2.3 Fluorography

Radioactive proteins resolved within SDS-PAGE gels were displayed by fluorography (Bonner & Laskey, 1974). In brief, gels were equilibrated in 500 ml glacial acetic acid for 20 min, followed by equilibration in 20% (w/v) 2,5-diphenyloxazole in glacial acetic acid for 20 min. The gels were washed in running tap water for 20 min and then dried on to a sheet of Whatman 3MM cellulose chromatography paper. Dried gels

were mounted on card and exposed to Kodak BIOMAX-MR film at -70 °C for 24-72 hr. Developed fluorograms were scanned by a 2202 Ultroscan laser densitometer linked to an LKB Bromma 2220 Recording Integrator to determine relative intensities of bands.

2.2.4 Electroblotting of Proteins to PVDF Membrane for Sequence Analysis

To aid blotting the separating and stacking gels were cast 16 hr prior to use, and then pre-run with separating gel buffer (appendix 2) containing 0.1 mM thioglycolic acid, to ensure free -arcylamide and -radicals would not be available to N-terminally block proteins.

After electrophoresis, the contents of the gel were transferred to PVDF (Problott) using a Trans-Blot electrophoretic transfer cell apparatus (Bio-Rad). For analysis of proteins below 50-60 kDa (two dimensional analysis of C-proteinase, Section 2.8.3), a 25 mM Tris-HCl buffer, 190 mM glycine in 10 % (v/v) methanol transfer buffer was used. Whereas for proteins greater than 50 kDa (chick C-proteinase analysis, Section 2.7 and type I procollagen cleavage products, Section 2.8.1), a 10 mM CAPS, pH 11, and 10% (v/v) methanol transfer buffer was used. A gel/ PVDF/ blotting paper sandwich was prepared as follows. PVDF membrane, the same size as the gel, was soaked in 10% (v/v) methanol for 10 sec, equilibrated in 100 ml transfer buffer and placed on to three pieces of Whatman 3MM blotting paper also soaked in transfer buffer. The SDS-PAGE gel, equilibrated in 200 ml of transfer buffer, was placed on to the membrane, and a further three more pieces of blotting paper placed above that.

Whilst preparing the sandwich, all air bubbles were removed. The apparatus was assembled and placed into the transfer unit cooled to ~2 °C. A current of 300 mA was passed through the cooled transfer system for 2 hr to ensure transfer was complete.

After completion of protein transfer the membrane was rinsed in water, saturated in 100% (v/v) methanol for a few seconds, proteins were then stained with 200 ml of 0.1% (w/v) Coomassie Blue in 40% (v/v) methanol and 1% (v/v) glacial acetic acid, for ~2 min. The PVDF membrane was removed from the stain, destained in 50% (v/v) methanol with frequent changes, and rinsed extensively in water. Membranes were air-dried overnight, placed in a plastic bag and stored at 4 °C. Bands of interest were excised for N-terminal sequence analysis.

2.3 CELL CULTURE

MG63 human osteosarcoma cells were obtained frozen, after 107 passages, from the European Collection of Animal Cell Cultures (ECACC Number: 86051601). The cells were maintained in Costar 162 cm^2 flat tissue culture flasks with 25 ml Dulbecco's Modified Eagles Medium (DMEM), containing 10% (v/v) heat inactivated foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (DMEM complete medium). Cells were passaged twice a week in a ratio of 1:3, to ~1 x 10^6 cells/flask, with the addition of 4 ml of 0.25% (w/v) trypsin per flask,.

2.3.1 Revival from Frozen Stocks

Cells stored under liquid nitrogen, in 1 ml cryogenic vials, were thawed rapidly under a stream of warm water, and gently transferred to a 75 cm^2 tissue culture flask, containing 20 ml of DMEM complete medium, and incubated at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After incubating for 72h, cells had reached 80-90% confluency (~1.5 x 10^6 cells), and were subcultured to a 162 cm^2 flask in 25 ml of DMEM complete medium.

2.3.2 Determination of Cell Concentration and Viability

Cell samples were diluted with an equal volume of 0.5% (w/v) trypan blue in phosphate buffered saline (PBS), and counted using an haemocytometer (Improved Neubauer). Viable cells that excluded trypan dye appeared clear and dead cells appeared blue. An average of two counts of viable cells was used to calculate total cell number.

2.4 PURIFICATION OF TYPES I AND III PROCOLLAGEN

2.4.1 Type I Procollagen From Chick Embryo Tendons (CET)

Type I procollagen was radiolabelled with mixed ^{14}C-amino acids and purified from matrix free cells from 17 day chick embryo leg tendons (Tuderman *et al.*, 1978; Prockop & Tuderman, 1982). Leg tendons (32.8 g) from 50 dozen chick embryos were digested with collagenase (9.4 mg/g tendon) and trypsin (8.2 mg/g tendon) for 2 hr at 37 °C under 10% CO_2 and 90% air. Krebs buffer (Appendix 3), supplemented

with 10% (v/v) FCS (FCS-Krebs) was added to the tendons and centrifuged at 300 x g, 500 x g, 600 x g and 1800 x g for 3, 5, 6, and 10 min respectively, with fresh buffer being added after each spin. The pellet of cells was resuspended in FCS-Krebs buffer, and centrifuged at 1800 x g for 2 x 10 min, and 5 min. The cells were resuspended in 40 ml of FCS-Krebs and counted using a haemocytometer. Cells at a final concentration of 1 x 10^8/ml were incubated for 4 hr at 37 °C in Krebs buffer supplemented with ^{14}C-labelled mixture of L-amino acids (1 μCi/ml), 25 μg/ml ascorbic acid, 100 U/ml penicillin, and 100 μg/ml streptomycin.

After incubation, the cells were centrifuged at 1500 x g for 10 min and a proteinase inhibitor cocktail added (to final concentrations of 0.25 M EDTA, 10 mM N-ethylmaleimide, 10 mM p-aminobenzamidine and 1 mM phenylmethylsulfonyl (PMSF). Proteins were precipitated by adding 176 mg/ml ammonium sulphate (Fielder-Nagy, 1981) and incubating overnight at 4 °C. The sample was centrifuged at 10 000 x g for 1 hr and the pellet resuspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl, and stirred at 4 °C for 4 hr.

The following steps were carried out at 4 °C or on ice, and all the buffers contained 0.02% (w/v) NaN_3 as a bacteriostat. To purify type I procollagen, the solution was dialysed against 2 x 2 L of DEII buffer, consisting of 0.075 M Tris-HCl buffer, pH 7.8, containing 2 M urea for a total of 24 hr, and the dialysate (45 ml) loaded on to a 1.5 cm x 5 cm DEAE-cellulose column, equilibrated with DEII buffer at a flow rate of 60 ml/hr. The column was washed with 30 ml of DEII buffer and bound proteins, including type I procollagen, were eluted with a linear NaCl gradient (480 ml, 0 to

0.12 M) at a flow rate of 60 ml/hr. Five ml fractions were collected, and 50 µl from alternate fractions were added to 4 ml of liquid scintillant and radioactivity counted in a liquid scintillation counter. Fractions containing the procollagen were pooled and dialysed against 3 x 2 L of storage buffer consisting of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl for a total of 36 hr. The dialysate was concentrated from 130 ml to 3.5 ml by pressure ultrafiltration with an Amicon YM100 membrane and then stored at -20 °C.

2.4.2 Purification of Human Types I and III Procollagen

Types I and III procollagen were purified from the medium of cultured MG63 osteosarcoma cells and radiolabelled with ^{14}C-mixed amino acids. In brief, MG63 cells were grown to confluency at 37 °C in DMEM complete medium in 16 x 175 cm^2 tissue culture flasks. Procollagens were radiolabelled by incubating the cells with 15 ml/flask of DMEM complete medium, containing ^{14}C-labelled mixture of L-amino acids (1 µCi/ml) and 25 µg/ml ascorbic acid for 24 hr. The medium was harvested, and procollagens were labelled for a further 24 hr. The medium was then replaced with 15 ml/flask of DMEM complete medium, containing 25 µg/ml ascorbic acid without ^{14}C-radiolabel.

The following steps were carried out at 4 °C or on ice, and all the buffers contained 0.02% (w/v) NaN_3 as a bacteriostat. A 0.1 volume of 1 M Tris-HCl buffer, pH 7.4, containing 0.25 M EDTA, was added to each round of medium collected, and incubated on ice for 30 min. To precipitate the procollagens 176 mg/ml solid

ammonium sulphate was added and stirred overnight at 4 °C. To collect the precipitated procollagens the rounds of medium were centrifuged at 10 000 x g and 4 °C for 60 min. The pellets were then stored at -20 °C.

Precipitated proteins from the three rounds were resuspended into a final volume of 50 ml of storage buffer and stirred overnight. The combined suspension was centrifuged at 20 000 x g and 4 °C for 30 min and the supernatant dialysed against 2 x 2L of DEI buffer, consisting of 0.1 M Tris-HCl buffer, pH 7.8, containing 2 M urea and 0.2 M NaCl, for a total of 24 hr. The dialysate was loaded on to a 1.5 cm x 10 cm DEAE-cellulose column equilibrated with DEI buffer, at a flow rate of 60 ml/h. The column was washed with 40 ml of the same buffer and 5 ml fractions collected. Radioactivity in alternate fractions was determined by liquid scintillation counting (LSC) of 100 µl aliquots. Fractions containing 95% of the eluted protein were pooled (70 ml), dialysed against 2 x 2 L of DEII buffer for a total of 24 hr, and loaded on to a 1.5 cm x 5 cm DEAE-cellulose column equilibrated with DEII buffer, at a flow rate of 60 ml/hr. After the column was washed with 50 ml of DEII buffer, the procollagens were eluted with a linear NaCl gradient (480 ml, 0-0.12 M) at a flow rate of 60 ml/hr. Aliquots from alternate fractions were analysed by LSC and SDS-PAGE (3.5% (w/v) polyacrylamide stacking and 7% (w/v) polyacrylamide separating gels, Appendix 2), to determine which fractions contained the procollagens.

Fractions containing type I procollagen were pooled and dialysed against 2 x 2 L of storage buffer for a total of 24 hr. The dialysate was concentrated by ultrafiltration with an Amicon 43 mm YM100 membrane and stored at -20 °C. For procollagen

cleavage studies (Section 2.8.1) a 1.2 ml sample of type I procollagen (~150 μg) was concentrated to 160 μl using a Centricon cell (Amicon) fitted with a YM100 membrane and centrifuging at 1000 x g and 4 °C for 1.5 hr.

Fractions containing type III procollagen were pooled and dialysed against 2 x 2L of 0.05 M sodium cacodylate, pH 7.4, containing 0.2 M NaCl and 25 mM EDTA, for a total of 24 hr. The dialysate was concentrated from 120 ml to 2.5 ml by ultrafiltration using an Amicon YM100 membrane and stored at -20 °C.

The procollagen concentration was determined by a colorimetric assay (Woessner, 1961), assuming that hydroxyproline constitutes 10.1% of type I procollagen weight (Fielder-Nagy *et al.*, 1981) (Appendix 4).

2.5 ASSAY OF PROCOLLAGEN C-PROTEINASE

An assay measuring rate of cleavage of the C-propeptide from procollagen proα chains developed by Kessler *et al.*, was modified into an electrophoretic detection assay (Kessler & Goldberg, 1978; Hojima *et al.*, 1985). On ice, the sample (up to 90 μl) to be assayed was added to 1μl ^{14}C-CET type I procollagen (1μg). Solutions were added to make a final of 0.05M Tris-HCl buffer, pH 7.5, containing 0.15 NaCl and 5 mM $CaCl_2$, and a final volume of 100 μl, and the mixture incubated for 2 min to 6 hr at 37 °C. The reaction was stopped with the addition of 10μl of 0.2 M EDTA, and the samples prepared for analysis by SDS-PAGE under reducing conditions, using a 3.5% (w/v) polyacrylamide stacking gel and a 7% (w/v) polyacrylamide separating gel

(Appendix 2). Dried gels were analysed by fluorography (Section 2.2.3). The initial rate of cleavage of procollagen to pN-collagen was determined by plotting the % cleavage of proα1(I) against time, where the % cleavage was determined by manipulation of the relative intensities of the proα1(I) and pNα2(I) bands (Equation 1), as determined by laser densitometry (Section 2.2.3). Units of enzyme activity were then calculated where 1 unit of activity was the amount that converts 1 μg of procollagen to pNα2(I) in 1 hr at 37 °C, at a procollagen concentration of 12 μg/ml.

$$\frac{\left[pN\alpha 2(I) \cdot \frac{150}{116}\right]}{\left[pN\alpha 2(I) \cdot \frac{150}{116}\right] + \left[\frac{pro\alpha 1(I)}{x}\right]} \times 100 = \%\ cleavage$$

Equation 1 Calculating % Cleavage in the Electrophoretic Assay

pNα2(I) and proα1(I) values are the areas of intensity of each band
150 and 116 are values allowing for differences in molecular weight (Kadler & Watson, 1995)
x is the ratio of Proα1(I):Proα2(I) in the uncleaved substrate

2.5.1 Development of an Electrophoretic Assay of C-proteinase with Enhanced Sensitivity

Addition of the neutral polymers, dextran sulphate (DS) and polyethylene glycol (PEG), to cultures of dermal fibroblasts induces complete processing of type I procollagen to collagen (Bateman *et al.*, 1986), and precipitation of types I, II and III collagen respectively (Ramshaw *et al.*, 1984). Hojima *et al.*,. aggregated type I

procollagen with these polymers, and rates of cleavage, by purified and crude C-proteinase, increased 10-15 fold with 0.02-0.05% (DS), and 2-20 fold with 2-4% PEG (Hojima *et al.*, 1994). Identical samples were assayed in the presence of 1 μg ^{14}C-CET procollagen, and 0.02-0.05 %(w/v) 500 kDa DS or 2-5% (w/v) PEG (10 kDa) or buffer, and incubated at 37 °C in a final buffer as described in section 2.5. The assay was stopped and samples analysed, as before in section 2.5. These studies were modified to develop a more sensitive assay, used to detect low levels of C-proteinase in cell culture studies and in fractions eluted from column chromatography.

2.5.1.1 C-proteinase detection in chromatography fractions and media using the enhanced assay

Samples were assayed for C-proteinase, with the addition of 0.02% (w/v) DS, as this was shown to enhance cleavage of procollagen by ~2-4 fold, whereas PEG had no effect. In brief, 1 μg ^{14}C-CET procollagen was incubated for 4 hr at 37 °C, with 89 μl of media or 5-10 μl of chromatography fraction (in a final of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 0.02% (w/v) 500 kDa DS and 5 mM $CaCl_2$) allowing cleavage to go to completion. Samples were analysed by SDS-PAGE, consisting of a 3.5% (w/v) polyacrylamide stacking gel and a 7% (w/v) polyacrylamide separating gel (Appendix 2).

2.6 PURIFICATION OF PROCOLLAGEN C-PROTEINASE

Chick and human procollagen C-proteinases were purified using a protocol devised by Hojima *et al.*, with minor modifications (Hojima *et al.*, 1985; Kadler & Watson, 1995).

With exception of the preparative step of the cultured media, all procedures were performed at 4 °C or on ice. All buffers contained 0.02% (w/v) NaN_3, as a bacteriostat, and their pH adjusted at room temperature. The electophoretic assay was used to detect C-proteinase throughout purification, and protein concentration determined by optical absorbance, using a Pharmacia UltrospecIII UV/visible spectrophotometer, at a wavelength of 280nm (where 1 absorbance unit was assumed to be equivalent to 1 mg of protein). Chick C-proteinase was purified from organ cultures of leg tendons from 17 day old chick embryos, and human C-proteinase from harvested media, from MG63 human osteosarcoma cells. The following two sections describe how cultured media were prepared for column chromatography.

2.6.1 Preparation of Crude Cell Cultures

2.6.1.1 Organ culture of chick embryo leg tendons

Leg tendons (20-25g) removed from 50 dozen 17 day old chick embryos were washed in Krebs glucose solution (Appendix 3) removing all traces of blood. Tendons were split into two 1 L Erlenmeyer flasks and gassed for several minutes with 5% CO_2 and 95% air, and cultured for 8 hr at 30 °C in 600 ml of DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin. Medium was collected from the tendons and replaced with a further 200 ml for two more 8 hr incubations at 30 °C. Harvested cultured medium (1 L) was then centrifuged at 600 x g for 3 min at room temperature and stored at -20 °C.

2.6.1.2 Cell culture of MG63 cells

MG63 human osteosarcoma cells were maintained as described in section 2.3. Cells were grown to confluency in 20 x 162 cm^2 flasks, in 25 ml/flask DMEM complete medium. The medium was removed by aspiration every 4 days, frozen at -20 °C and replaced with a further 25 ml. Each flask was maintained for 4 weeks. A total of 6.6 L of medium was harvested and used in protein purification as described in section 2.6.2. To ensure active enzyme was being secreted into the media throughout the 4 weeks, each harvest was assayed for C-proteinase using the enhanced electrophoretic assay (Section 2.5.1.1).

2.6.2 Stages of Purifying Chick and Human C-proteinase

2.6.2.1 Preparation of frozen chick and human culture medium

Frozen medium from either chick or human source was thawed at 4 °C and the pH adjusted to 7.5. Tris-base, NaCl and NaN_3 were added to final concentrations of 0.05 M, 0.3 M and 0.02% (w/v) respectively, and pH adjusted to 8-8.1. The supernatant was collected after centrifuging the sample at 17 000 x g for 30 min at 4 °C.

2.6.2.2 Green A dye matrex gel chromatography

The supernatant, from the preparative media, was applied to a 5 cm x 10 cm column of Green A DyeMatrex gel, equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl (equilibration buffer), at a flow rate of 120 ml/hr. The column was washed with 300 ml of equilibration buffer, and then 800 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl. Absorbed proteins, including C-proteinase, were

eluted with 600 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 3 M NaCl and 2 M urea (elution buffer). Samples (5 µl) from alternative fractions were assayed for C-proteinase, using the electrophoretic assay. Fractions containing enzyme activity were pooled and concentrated to ~60 ml by pressure ultrafiltration in an Amicon 350 ml chamber, with a 72 mm YM30 membrane. After concentrating for 14 hr the sample was stirred for 2 hr, removed and then the membrane was washed with 10 ml of elution buffer for a further 2 hr. The sample and wash were combined, and activity determined using the enhanced electrophoretic assay. The sample was diluted with an equal volume of 0.15 M Tris-HCl buffer, pH 7.5, containing 2 mM $CaCl_2$ giving final concentrations of 0.1 M Tris-HCl buffer, pH 7.5, 1.5 M NaCl, 1 M urea and 1 mM $CaCl_2$. The solution was centrifuged at 15 000 x g for 60 min and the supernatant collected.

2.6.2.3 Concanavalin A-Sepharose chromatography

The supernatant, from the Green A DyeMatrex chromatography, was applied to a 1.5 cm x 11 cm, column of concanavalin A conjugated to Sepharose, equilibrated with 0.1 M Tris-HCl buffer, pH 7.5 containing 1.5 M NaCl, 1 M urea and 1 mM $CaCl_2$ (equilibration buffer) at a flow rate of 20 ml/hr. The column was washed with 60 ml of equilibration buffer at a flow rate of 20 ml/hr and C-proteinase was eluted with 400 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.7 M α-methyl-D-mannoside, 1.5 M NaCl and 1 M urea (elution buffer), at a flow rate of 5 ml/hr. Samples (5 µl) from eluted fractions were assayed for C-proteinase using the electrophoretic assay. Fractions containing C-proteinase were pooled and concentrated for 8 hr to 30-35 ml

using an Amicon YM30 62 mm membrane in a 200 ml chamber. After concentrating, the sample was stirred for 1 hr, removed and then the membrane washed twice with 2 ml of elution buffer for a further 2 hr. To the combined samples Brij-35 was added to a final concentration of 0.005% (w/v). The sample was dialysed against 2 x 2 L of 0.3 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl and 0.005% (w/v) Brij-35 for a total of 22 hr, and the dialysate centrifuged at 17 500 x g for 25 min, and the supernatant collected. Units of C-proteinase were determined by assaying 5 µl of the supernatant.

2.6.2.4 Heparin-Sepharose chromatography

The supernatant, from the concanavalin A-Sepharose chromatography, was applied to a 2.5 cm x 10 cm column of heparin conjugated to Sepharose, equilibrated with 0.3 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl and 0.005% (w/v) Brij-35 (equilibration buffer), at a flow rate of 25 ml/hr. Proteins were washed off the column with equilibration buffer (protein peak a) and eluted with 0.3 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.005% (w/v) Brij 35 (elution buffer, protein peak b). To clean the column, all other proteins were eluted with 0.3 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, 1 M urea and 0.005% (w/v) Brij 35 (cleaning buffer). Samples (10 µl) from fractions from both E-A and E-B peaks were assayed for C-proteinase using the enhanced electrophoretic assay. Both peaks contained active enzyme; the 'a' protein peak contained enzyme-A (termed E-A) and the 'b' protein peak contained enzyme-B (termed E-B). E-A and E-B were pooled and stored separately. To the enzyme solution NaCl, urea and $CaCl_2$ were added to final

concentrations of 1 M, 1 M and 5 mM respectively. Samples were then concentrated to ~6 ml in an Amicon 200 ml chamber, with a 62 mm YM30 membrane. After concentrating for 5 hr the sample was stirred for 1 hr, removed and the membrane washed with 2 x 1 ml of cleaning buffer for a total of 2 hr. Samples were combined and activity determined in 10 µl samples using the C-proteinase electrophoretic assays.

2.6.2.5 Sephacryl S-300 gel filtration

Concentrated E-A or E-B from the heparin-Sepharose column, was applied to a 2.5 x 110 cm column of Sephacryl S-300, equilibrated with 0.3 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl, 5 mM $CaCl_2$ and 0.005% (w/v) Brij 35, at a flow rate of 15 ml/hr. Samples (5 µl) from fractions were assayed for C-proteinase, and active fractions pooled, urea and α-methyl-D-mannoside were then added to final concentrations of 1 M and 0.2 M respectively. The sample was concentrated to ~1.5 ml by ultrafiltration using an Amicon YM30 43 mm membrane. After concentrating for 2 hr the sample was left stirring for 1 hr and the membrane washed with 2 x 0.5 ml of 0.3 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, 1 M urea and 0.005% (w/v) Brij 35. Enzyme activity was determined and samples were then frozen in aliquots at -20 °C.

2.7 SEQUENCE ANALYSIS OF CHICK C-PROTEINASE

To concentrate chick C-proteinase 1 ml (0.5 mg protein) of E-A, from the heparin-Sepharose column was dialysed against 2 x 2 L of 0.1 M glacial acetic acid for a total of 16 hr, and the dialysate freeze dried overnight in two eppendorfs. The dried

samples, in each eppendorf were resuspended in 50 µl of 0.125 M Tris-HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue, and combined. The sample was reduced by heating at 100 °C for 3 min in the presence of 2% (v/v) 2-mercaptoethanol, and subjected to SDS-PAGE using a 7% (w/v) polyacrylamide separating gel overlaid by a 3.5% (w/v) polyacrylamide stacking gel (Appendix 2). Proteins in the gel were blotted on to PVDF, stained with Coomassie Blue, and three bands of interest were excised and N-terminally sequenced.

2.8 CHARACTERISATION OF HUMAN PROCOLLAGEN C-PROTEINASE

2.8.1 Cleavage of Type I Procollagen with Human C-Proteinase and Sequence Analysis of C-Propeptides

To obtain N-terminal sequences of cleaved C1 and C2 -propeptides from procollagen by human C-proteinase, ~100% procollagen was cleaved with DS, and the cleavage products blotted. In a final volume of 600 µl, 150 µg of ^{14}C-CET procollagen (150 µl) or ~110 µg human type I procollagen (180 µl) was incubated overnight at 37 °C with 6.6 units of human C-proteinase (E-A, 30 µl), in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.02% (w/v) 500 kDa DS, 0.15 M NaCl and 5 mM $CaCl_2$. The sample was then reduced and the C-propeptides separated on a pre-treated 15% (w/v) polyacrylamide gel (Appendix 2). Proteins in the SDS gel were then transferred to PVDF membrane by blotting, and the membrane exposed to X-ray film for 16 hr. Bands of the C1 and C2 -propeptides from the cleavage were carefully excised and the N-terminal sequence obtained. To ensure the proteins had been transferred to the membrane the gel was stained with Coomassie Blue.

2.8.2 Investigating Factors Affecting C-proteinase Activity

2.8.2.1 Class specific inhibitor assay

A number of inhibitors specific to classes of proteinases, metallo- (EDTA, *o*-phenanthroline), cysteine (N-ethylmaleimide) and serine (PMSF and Soybean trypsin inhibitor) were incubated with mixtures of human E-A to investigate their ability to inhibit human C-proteinase. Human E-A (2.5 µl, 0.5 unit) was added to the inhibitor and incubated in a volume of 50 µl at room temperature for 30 min. To the inhibitor reaction mixture 1 µl ^{14}C-CET procollagen (1 µg) was added with 49 µl of buffer making 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 5 mM $CaCl_2$. The samples were incubated at 37 °C for 40 min, reduced and subjected to SDS-PAGE using a 7% (w/v) polyacrylamide separating gel (Appendix 2). The gels were prepared for fluorography and analysed by densitometry (Section 2.2.3).

2.8.2.2 Effects of buffer components on activity

C-proteinase was incubated in the presence of materials used during chromatography to check for other specific affects on activity. Human E-A (2.5 µl, 0.5 unit) was added to 1 µl ^{14}C-CET procollagen (1 µg) in the presence of either 0.1-1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 5 mM $CaCl_2$, or 0.03 M Tris-HCl buffer, pH 7.5, containing 0.1-1 M NaCl and 5 mM $CaCl_2$, or 0.05 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl, 0.5-0.95 M α-methyl-D-mannoside and 5 mM $CaCl_2$. The samples were incubated at 37 °C for 4 hr, reduced and subjected to SDS-PAGE using

a 7% (w/v) polyacrylamide separating gel (Appendix 2). The gels were prepared for fluorography and analysed by densitometry (Section 2.2.3).

2.8.2.3 Human C-proteinase calcium dependency

Human E-B eluted from the heparin-Sepharose column was used in this investigation, as the elution buffer did not contain calcium. However, the sample taken was from the pool prior to concentrating, so to increase the sensitivity of the assay the enhanced assay was used. E-B (10 µl, ~0.005 units) was added to 89 µl of buffer containing 0.05 M Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 0.02% 500 kDa DS and $CaCl_2$ (0-50 mM), and 1 µl ^{14}C-CET procollagen (1 µg). The reaction mixture was incubated for 4 hr at 37 °C and reduced samples were subjected to SDS-PAGE using a 7% (w/v) polyacrylamide separating gel, and fluorography (Section 2.2.3).

2.8.2.4 Effects of freeze thawing on C-proteinase activity

Samples of human E-A (2.5 µl, 1.3 unit) were freeze thawed, by thawing at room temperature and freezing at -20 °C for a range of cycles (2-32). The samples were then assayed for C-proteinase activity, using the basic assay (Section 2.5), and samples were subjected to SDS-PAGE using a 7% (w/v) polyacrylamide separating gel, and fluorography (Section 2.2).

2.8.2.5 Effects of long term storage on C-proteinase activity

Chick E-A and E-B (5 µl samples) were assayed for C-proteinase, using the basic assay, under identical conditions before and after 2 years storage at -20 °C to determine stability. Samples were subjected to SDS-PAGE using a 7% (w/v) polyacrylamide separating gel, and fluorography (Section 2.2).

2.8.3 Two Dimensional Analysis of Cyanogen Bromide Fragments of Proteins in Human E-A and E-B Fractions

2.8.3.1 Concentrating human E-A and E-B

Proteins, including C-proteinase, in E-A and E-B from one purification, were radiolabelled with ^{14}C-formaldehyde and analysed by cyanogen bromide (CNBr) cleavage mapping in two dimensions (2D). Proteins in E-A (2.8 ml, ~12 µg) and E-B (10 ml, ~17 µg) were precipitated by adding 20% (w/v) trichloroacetic acid and incubated at room temperature for 4 hr. Precipitated proteins were collected by centrifugation at 9 000 x g for 1.5 hr and resuspended in 400 µl of 0.15 M sodium tetraborate and 20 mM sodium cyanoborohydride, pH 8.0. Samples were then radiolabelled as described in the following section.

2.8.3.2 ^{14}C-formaldehyde radiolabelling of proteins

Proteins to be radiolabelled (~400-600 µl) were dialysed against 2 x 2 L of 0.15 M sodium tetraborate and 20 mM sodium cyanoborohydride, pH 8.0 at room temperature for a total of 16 hr. To the dialysate 50 µCi of ^{14}C-formaldehyde was added and

incubated, shaking at room temperature overnight. Samples were then dialysed against an appropriate buffer. The human samples, for 2D CNBr fragment analysis, were dialysed against 10 x 100 ml of PBS for a total of 35 hr, and chick E-A and E-B dialysed against 2 x 2 L of 0.1 M sodium phosphate buffer, pH 7.0, for a total of 16 hr. Aliquots (5 µl) were taken and free radioactive counts determined by LSC.

2.8.3.3 Cyanogen bromide cleavage and 2D analysis

Radiolabelled human E-A and E-B samples (200 µl) were prepared for SDS-PAGE, under reducing conditions as described in section 2.2. The two samples were separated by a 9.5% (w/v) polyacrylamide separating gel overlaid by 3.5% (w/v) polyacrylamide stacking gel (Appendix 2) pre-treated as described in section 2.2.4. The lane containing the separated proteins was cut out, transferred to a 50 ml capped tube and 300 ml of 70% (v/v) formic acid added for 3 x 30 min. The formic acid was removed and the gel slice incubated under a nitrogen atmosphere in 10 ml in a 50 mg/ml CNBr, 70 % (v/v) formic acid solution (handled with extreme care and in a fume hood) for 3 hr at room temperature with frequent shaking. The CNBr solution was replaced with 40 ml of 1 M Tris-HCl buffer, pH 6.8, containing 0.001 % (w/v) bromophenol blue and incubated at room temperature for 2 x 30 min. Proteins were then reduced for 1 hr in 0.125 M Tris-HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) bromophenol blue and 2% (v/v) 2-mercaptoethanol. The reducing stain was removed and the gel slice equilibrated in the same stain without 2-mercaptoethanol for 2 hr with frequent changes.

To separate the CNBr fragments, the gel slice was carefully overlaid on to a 3.5% (w/v) polyacrylamide stacking gel and 15% (w/v) polyacrylamide separating gel (Appendix 2). A 3.5% (w/v) acrylamide stacking gel solution was added carefully around the gel slice, left to set, and the gel run at a constant current of 50 mA. CNBr fragments were blotted to Problott as described in section 2.2.4 and stained with Coomassie Blue. Fragments found at approximately the same molecular weight in both E-A and E-B samples were carefully cut out and N-terminally sequenced. After blotting the gel was dried and analysed by phosphoimaging.

2.9 REGULATION OF HUMAN PROCOLLAGEN C-PROTEINASE IN CULTURED MG63 CELLS

2.9.1 Effects of Seeding Density, Ascorbate, Volume of Media and DMEM on C-proteinase Detection

In Costar 6 well plates, cells were seeded at 0.75 and 1.5 x 10^5 cells per 9.6 cm^2 well in 1 ml of DMEM complete medium, and incubated at 37 °C in a humidified tissue culture incubator. After 24 hr to allow cell attachment, cell layers were washed twice with 2 ml of either FCS free -DMEM or -MEM complete medium containing 1% (v/v) Nutridoma and 0 or 50 μg/ml ascorbate (test medium). The medium was removed by aspiration and replaced by 1 or 4 ml of fresh test medium, in triplicate, and incubated for 1, 3 and 5 days. At these time points, the medium was removed and assayed (89 μl) for C-proteinase activity using the enhanced electrophoretic assay, and the cells were counted using a haemocytometer with 0.25% (w/v) trypan blue.

2.9.2 Growth Factors

Recombinant human FGFb, IL-1β and IL-4 were reconstituted in 1 ml PBS containing 0.1% (w/v) bovine serum albumin (BSA). Recombinant human TGFβ1 was reconstituted in 1 ml of 4 mM HCl containing 0.1% (w/v) BSA making a final stock of 2 μg/ml. Recombinant human EGF was reconstituted in 1 ml of 10 mM glacial acetic acid containing 0.1% (w/v) BSA making a final stock of 100 μg/ml.

2.9.3 Effects of growth Factors : Experimental Growth Conditions

MG63 cells seeded at 1.5 x 10^5 cells per 9.6 cm^2 well in 1 ml of DMEM complete medium containing 50 μg/ml ascorbate, were incubated for 24 hr to allow cell attachment at 37 °C in a humidified tissue culture incubator. The cells were washed twice with 2 ml of FCS free DMEM complete medium, containing 1% (v/v) Nutridoma and 50 μg/ml ascorbate (growth medium). Cells were incubated for 3 hr with 1 ml of growth medium, whereupon it was removed by aspiration and replaced with 1 ml of growth medium containing the indicated amounts of growth factors (as described below) and incubated for 1, 3 and 5 days after this point that was taken as day 0. Growth factors added to the cells were as follows: 0.1, 1, 10 ng/ml FGFb, 0.1, 1, 10 ng/ml TGFβ1, 0.5, 5 and 50 ng/ml EGF, 0.1, 1, and 10 ng/ml IL-4 and 0.5, 10 and 20 ng/ml IL-1β.

Control wells containing growth medium without the growth factor were included and parallel dishes in triplicate were set up to determine cell number (MTT assay, Section 2.9.3.1), collagen in the cell layer (Sircol Collagen assay, Section 2.9.3.2), and C-

proteinase activity in the medium (Section 2.5.1) at the 3 time points (a total of 9 wells/time point/concentration of growth factor).

2.9.3.1 Rapid colorimetric assay for cell growth

Cleavage of tetrazolium rings by the mitochondrial enzyme succinate-dehydrogenase (Slater *et al.*, 1963) formed the basis of an assay for adherent cell survival and proliferation. The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was cleaved into a blue-purple coloured product, formazan, by active succinate-dehydrogenase in living cells. Formazan was then quantified using a scanning multiwell spectrophotometer and the absorbance used to determine MG63 cell proliferation (Mossman, 1983; Denizot & Lang, 1986).

Medium was removed from each well by inverting, flicking and blotting the plate. To the cells in each well 100 µl of a sterile filtered 5 mg/ml solution of MTT in PBS was added. Plates wrapped in tin foil were incubated for 4 hr at 37 °C, and the formazan product solubilised by the addition of 1 ml 40 mM HCl in isopropanol, with shaking. The optical density of each well was measured using a Dynatech MR4000 plate reader with a test wavelength of 560nm against a blank containing solvent alone. A control of growth medium was used to determine actual absorbance. Linearity of the MTT assay was determined by plating out MG63 cells in doubling dilutions, with 0.1 ml growth medium, and MTT (10 µl of a 5 mg/ml stock), in triplicate in 96-well Nunclon plates. After 4 hr at 37 °C the formazan was solubilised, optical density read as described

above and a standard curve constructed (Appendix 5). Results were obtained by interpolation using this standard curve.

2.9.3.2 Sircol Collagen assay

The dye, Sirius Red F3BA was first used as a histochemical stain for collagen as an alternative to Van Giesons' picrofushin stain (Sweat *et al.*, 1964). It binds specifically to side chain groups of the basic amino acids present in collagen types I to V, as the elongated dye molecules align in parallel with the helical structure of collagen chains. A Sircol Collagen Assay kit (developed by Biocolor, N. Ireland) which incorporates Sirius red in a colorimetric reaction with collagen, was used in accordance to manufacturers instructions, to determine levels of insoluble collagen found in the cell layer.

The growth medium was removed by aspiration and cells, in triplicate wells, washed twice with 4 ml of PBS, and each plate inverted, flicked and blotted to remove any excess. To the well 0.5 ml of 0.1% (w/v) Sirius Red in saturated piric acid was added and incubated at room temperature for 1 hr on a rotator to gently mix. Plates were inverted and flicked removing the dye reagent, and 0.5 ml of NaOH added to solubilise the collagen bound dye. The plates were left rotating for 30 min at room temperature, and the optical density of the dye solution measured using a Pharmacia Ultrospec III at a test wavelength of 540nm with water as a reference. Reagent blanks were included in triplicate and this absorbance subtracted from test samples.

Linearity of the Sircol Assay was determined by assaying type I collagen standards in triplicate. To eppendorfs, 6.25, 12.5 and 25 µl of 1 mg/ml type I collagen in 0.5 M acetic acid were added, in a final sample volume of 100 µl 0.5 M glacial acetic acid, to 0.5 ml of Sirius dye reagent. After rotating for 1 hr at room temperature the samples were centrifuged at 5 000 x g for 5 min, and unbound dye solution removed by careful inversion and blotting with tissue. After eppendorfs were left inverted for 10 min, the bound dye was solubilised by the addition of 0.5 ml of 0.5 M NaOH, and optical density determined as described above. Reagent blanks were subtracted from absorbances of collagen standards and the standard curve constructed (Appendix 6). Results were obtained by interpolation using this standard curve.

2.10 STATISTICAL ANALYSIS

2.10.1 Hypothesis Testing

Combined experimental data from a minimum of three experiments are presented as the mean ± standard error of the mean (SEM), where SEM is the standard deviation $(s)/\sqrt{n}$. The Students Hypothesis test (Rees, 1985) for the difference in means of two populations (also called unpaired samples data) was used to determine statistical significance. A null hypothesis was set where there is no difference between the means (µ) of the two sets of data (H_0: $\mu_1=\mu_2$), and the alternative hypothesis set where there is a difference in either direction (a two-sided hypothesis).

$$Calc\ t = \frac{x_1 - x_2}{s\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where x= calculated mean

n= number of data points and,

$$s^2 = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1+n_2-2}$$

The calculated value of t was compared to tabulated t values and findings were considered significant if $p<0.025$ (for a two sided hypothesis).

2.10.2 Regression Analysis

Regression analysis was used to obtain the linear regression equation of a line of best fit, when two sets of data were graphically represented as a scatter diagram. The linear regression equation was in the form

$$y = a + bx$$

where b represents the gradient of the line and a represents the intercept

the values of a and b were calculated using

$$b = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}} \quad \text{and} \quad a = \bar{y} - b\bar{x}$$

where n= number of data points and,

where $\bar{x}$ and $\bar{y}$ are the sample means of x and y

The degree of association (correlation coefficient) between the two sets of data was determined by using the Pearson's correlation coefficient equation, where it was assumed that the variables were normally distributed (Rees, 1985).

$$r = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sqrt{\left[\sum x^2 - \frac{(\sum x)^2}{n}\right]\left[\sum y^2 - \frac{(\sum y)^2}{n}\right]}}$$

where r= correlation coefficient value

x= one variable and y= the other variable

3. RESULTS, PART I: PURIFICATION OF C-PROTEINASE

3.1 PURIFICATION OF PROCOLLAGENS

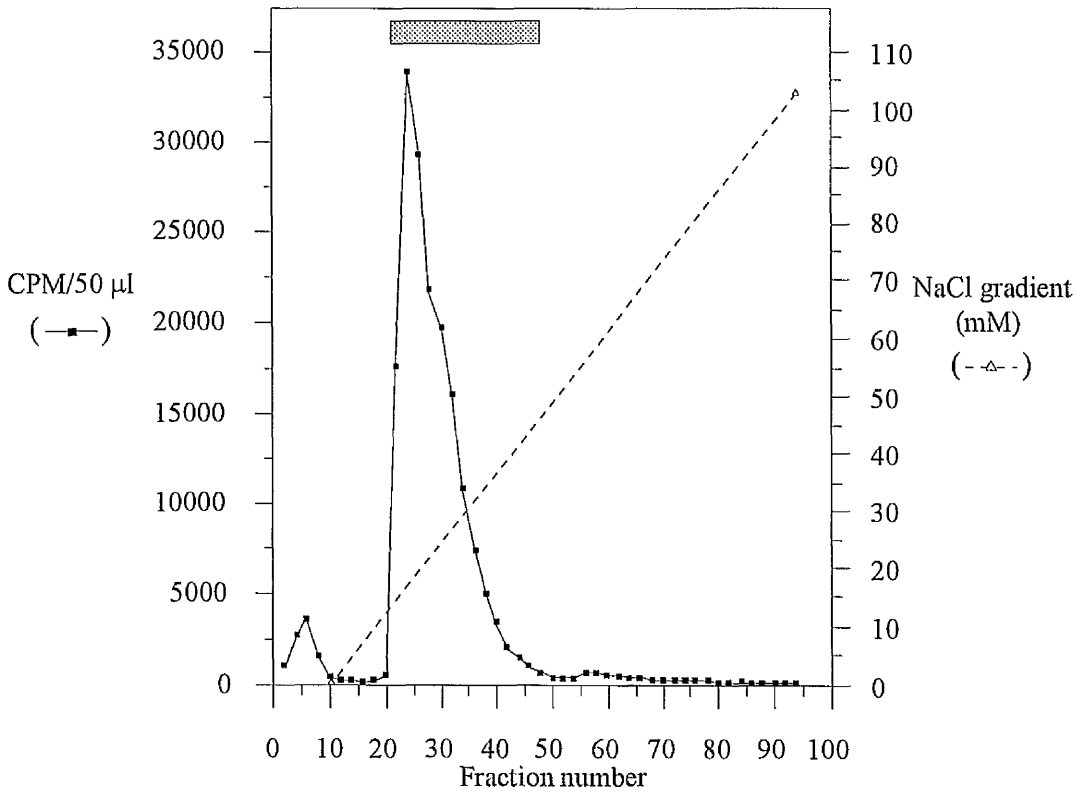
3.1.1 Chick Type I Procollagen

Fifty dozen 17 day old chick embryos were dissected, and 32.8 g of leg tendons removed, washed, digested with collagenase and trypsin, and 5.5 x 10^9 cells labelled with ^{14}C-mixed amino acids. Ammonium sulphate precipitated proteins were resuspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.4 M NaCl. After dialysing the sample against DEII buffer 45 ml of sample (total count = 4 x 10^7 CPM) was loaded on to a DEII column (Figure 3.1). Aliquots (50 µl) from alternate fractions were measured by LSC, and the radiolabelled protein peak (140 ml), eluted typically at a NaCl concentration of 10-15 mM, was pooled (total radioactive count 3.6 x 10^7 CPM). Recovery of protein was 81%, with 20% of proteins unbound to the column. After concentrating the sample to 3.5 ml (total radioactive count 3.19 x 10^7 CPM) with a recovery of 71%, the purified procollagen was visualised by SDS-PAGE and fluorography (Figure 3.2, lane 1). The expected 2:1 ratio of proα1(I):proα2(I) was observed, and cleavage of proα2(I) to pNα2(I) with chick C-proteinase is clearly seen. There is a possibility of proα1(III) also being included in the proα1(I) band (Figure 3.2). Ten microlitre samples were analysed in triplicate using the hydroxyproline assay (Appendix 4), determining the final procollagen concentration as 1 µg/µl.

3.1.2 Human Types I and III Procollagen

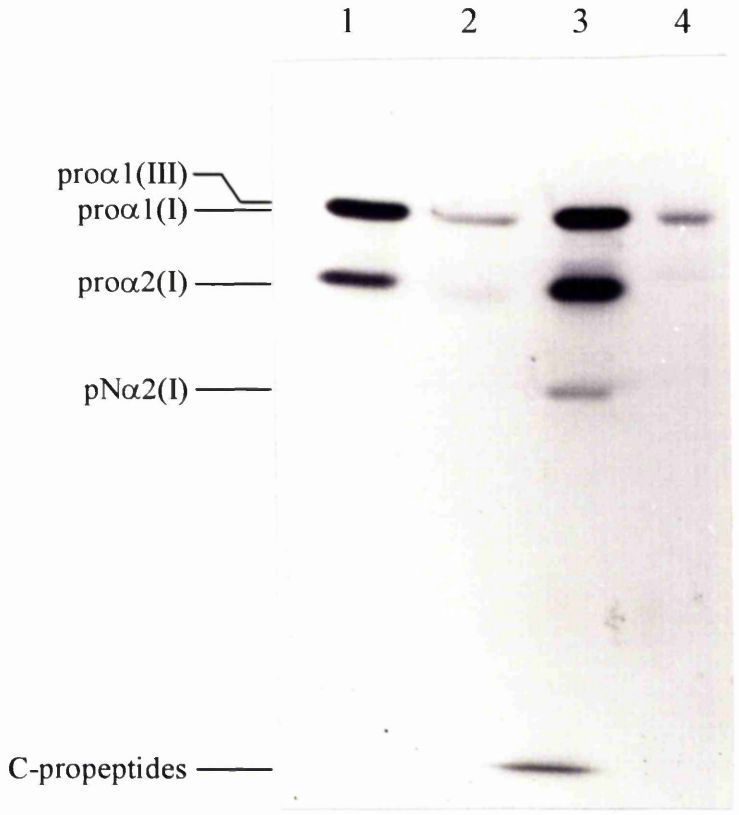
Human MG63 osteosarcoma cells grown in 16 x 175 cm^2 flasks were radiolabelled with ^{14}C-mixed amino acids for 2 x 24 hr, and 1 x 24 hr without label. Pellets of

Figure 3.1 DEAE-Cellulose Chromatography of Chick Type I Procollagen



Crude procollagen fractions were dialysed into DEII equilibration buffer and applied to a DEAE-Sepharose column (1.5 cm x 5 cm) and the column washed with equilibration buffer at a flow rate of 60 ml/hr. Bound proteins were eluted with a linear NaCl gradient (0-0.12M, shaded line) and 5 ml fractions collected. Radioactivity was determined by LSC of 50 µl samples from alternate fractions. Type I procollagen was eluted at 14 mM NaCl and fractions 21-48 (shaded box) were pooled.

Figure 3.2 Analysis of Purified Types I and III Procollagens by SDS-PAGE

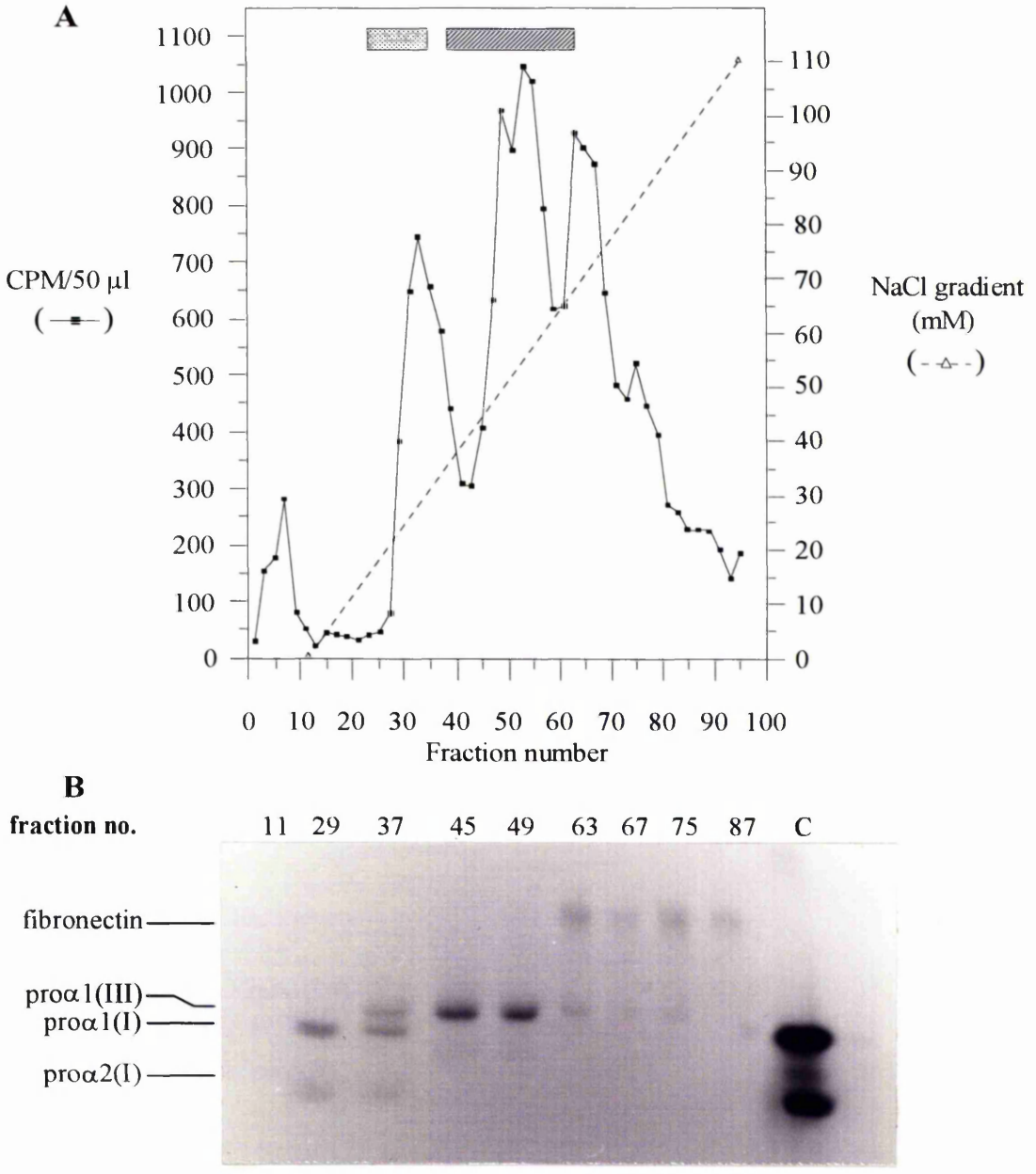


Purified procollagen samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and detected using fluorography. ***Lane 1,*** 1 µl of CET-procollagen (1 µg); ***Lane 2***, 4 µl of human type I procollagen (0.52 µg) from MG63 cells; ***Lane 3***, 1 µg CET-procollagen incubated with 1.5 units of chick C-proteinase (E-A) for 2 hr at 37 °C. ***Lane 4***, 2 µl human type III procollagen (~0.8 µg).

precipitated protein, from each round of collected media (~240 ml), were resuspended into a total of 50 ml of storage buffer, dialysed against DEI buffer, and the dialysate (55 ml, total radioactive count ~8.1 x 10^6 CPM) loaded on to a DEAE-Sepharose column equilibrated with DEI buffer. The radiolabelled protein peak in the unbound fractions (fractions 3-13) was pooled (67 ml, total radioactive count ~7.76 x 10^6 CPM), dialysed against DEII buffer, and the dialysate (~89% of radiolabelled protein, total radioactive count 7.25 x 10^6 CPM) loaded on to a DEAE-Sepharose column equilibrated with DEII buffer (Figure 3.3, A). The column was washed with 40 ml of DEII buffer, and the NaCl gradient applied (fraction 11 onwards). Aliquots (50 µl) from alternate fractions were measured by LSC (Figure 3.3, A), and samples from alternate fractions were visualised by SDS-PAGE and fluorography.

Type I procollagen eluted between 15 and 33 mM NaCl (fractions 28-35), and type III between 38 and 67 mM NaCl (fractions 38-63) were pooled separately (Figure 3.3, B), and dialysed. Fractions 36 and 37 were not pooled as they contained both types I and III procollagen. Type I procollagen was concentrated to 1.75 ml with a final count of 3.50 x 10^5 CPM (a recovery of 48% from the pool, and a 4.3% final recovery from initial radiolabelling). Type III procollagen was concentrated to 2.5 ml with a final count of 1.13 x 10^6 CPM (a recovery of 61.6% from the pool, and a 14% final recovery from initial radiolabelling). Final procollagen concentrations were determined, using the hydroxyproline assay, as 0.13 µg/µl and 0.41 µg/µl for type I and type III procollagen respectively. Purified human procollagens were visualised by

Figure 3.3 DEAE-Cellulose Chromatography of Human Types I and III Procollagen



A, Pooled proteins from the first DEAE-cellulose column were equilibrated with DEII buffer and applied to a second DEAE-cellulose column equilibrated in DEII buffer. Bound proteins were eluted with a linear NaCl gradient (0-0.12M, dotted line), and 5 ml fractions collected. Radioactivity was determined by LSC of 50 µl samples from alternate fractions. ***B***, Samples from fractions were analysed by SDS-PAGE, under reducing conditions, and proteins visualised by fluorography. Equivalent amounts of radioactivity (50 CPM) was loaded on to each lane. Fractions 28-35 (38 ml, shaded box), containing type I procollagen, and fractions 38-63 (126 ml, hatched box), containing type III procollagen were pooled respectively.

SDS-PAGE and fluorography (Figure 3.2, lanes 2 and 4). The proα1(III) chains migrate slower than the proα1(I) of type I procollagen.

3.2 ASSAY OF PROCOLLAGEN C-PROTEINASE

In the laboratory, C-proteinase was assayed using a 'rapid assay'. In the rapid assay C-proteinase was incubated with procollagen, the reaction was stopped by the addition of EDTA and 25% (v/v) cold ethanol (-20 °C) was added to precipitate uncleaved procollagen. The excised ^{14}C-propeptide fragments (in the supernatant) were then measured (by LSC) and units of activity determined (Kessler and Goldberg, 1978).

An advantage of the rapid assay is its speed and apparent ease of use. However, it is insensitive to non-specific cleavage of procollagen (at the N-propeptide end of the molecule) and errors can arise when cleaved procollagen molecules are not all precipitated by ethanol. High background readings thus result. For these reasons, a SDS-PAGE assay was used to assay C-proteinase.

3.2.1 Electrophoretic Assay

An electrophoretic assay was devised where the substrate and enzyme were incubated under identical conditions, to the 'rapid assay', and the whole sample analysed, without the addition of ethanol, by SDS-PAGE under reducing conditions and fluorography. To illustrate the assay, concentrated human and chick procollagens where cleaved by 1.5 units of chick C-proteinase and analysed (Figure 3.2, lane 3). The 2:1 ratio of proα1(I):proα2(I) is again observed, and cleavage of proα2(I) to pNα2(I) is clearly

seen (Figure 3.2, lane 3). Cleavage of proα1(I) to pNα1(I) is masked as pNα1(I) and proα2(I) migrate a similar distance. Purified chick type I procollagen was used as the substrate for the C-proteinase assay throughout the study. The assay was used to detect C-proteinase in column chromatography fractions (purification) and in growth media from MG63 cells.

Units of activity were determined from the rate of proα2(I) cleavage to pNα2(I), where the reaction rate is linear with respect to added C-proteinase. To calculate this, an equation (Equation 1) was devised in which only the intensity of the proα1(I) band was used instead of the proα2(I) band, as the pNα1(I) merges into the latter. This was achieved by using the value of the ratio of proα1(I):proα2(I) to equate the actual proα2(I) intensity. The equation was used throughout the study to standardise enzyme activity values.

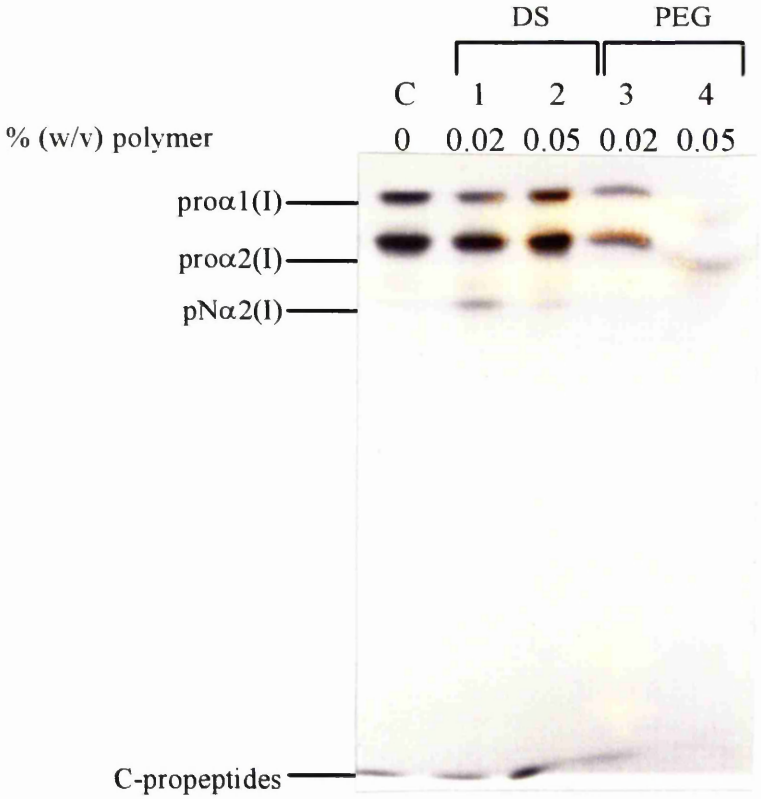
3.2.2 Development of an Enhanced Electrophoretic Assay

The electrophoretic assay was often not sensitive enough to detect the low levels of C-proteinase (if below 0.5 units/ml) present in chromatographic fractions, and in the growth media from MG63 cells. To increase the sensitivity of the assay, the polymers DS and PEG, were added to reaction mixtures to enhance cleavage, by aggregating procollagen (Bateman *et al*, 1986).

Media from human MG63 cells was assayed for C-proteinase with the addition of DS and PEG (Figure 3.4, A). The enzyme activity in the presence of 0.02% (w/v) DS

Figure 3.4 Effect of DS and PEG on Crude Human C-proteinase Cleavage of Procollagen

A



B

	% Cleavage
Control	5.5 ± 0.4
DS 0.02% (w/v)	18.4 ± 0.2
DS 0.05% (w/v)	10.3 ± 0.3
PEG 0.02% (w/v)	5.6 ± 0.2
PEG 0.05% (w/v)	5.2 ± 0.4

C-proteinase was assayed using the basic and enhanced assay (with DS), and analysed by SDS-PAGE (7% separating), under reducing conditions, and separated proteins detected by fluorography. ***A,*** crude MG63 media taken from cells growing in complete DMEM medium for 120 hr, in one 162 cm^2 flask, was assayed with the addition of polymers DS and PEG. ***Lanes 1-4***, MG63 media (60 µl) was added to 1 µg CET-procollagen with the indicated concentrations of DS and PEG, and incubated for 2 hr at 37 °C. ***Lane C***, media (60 µl) was added to 1 µg CET-procollagen and incubated without the polymers. ***B***, Percent cleavage of procollagen was determined using equation 1, the experiment was carried out in triplicate.

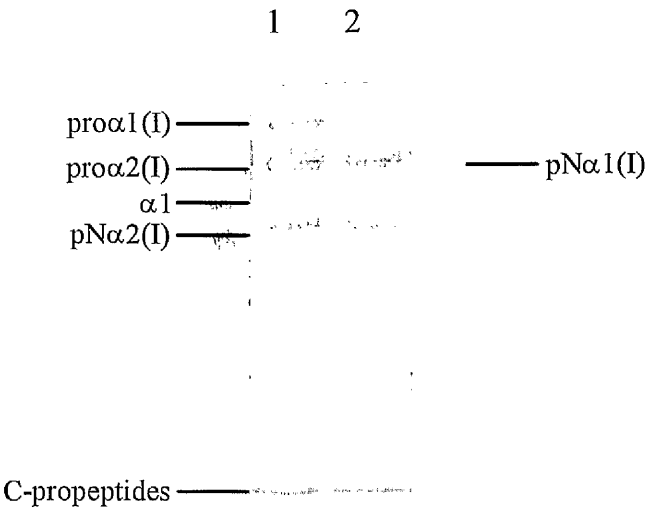
(Figure 3.4, B), increased from 5.5 to 18%, a 3.3 fold increment, 0.05% (w/v) DS had a less significant affect (2 fold increase in cleavage), and PEG had no affect. Purified C-proteinase from MG63 cells (Section 3.4), incubated with 0.02% (w/v) DS, was able to enhance cleavage of human procollagen from 40 to ~95%, a 2.4 fold increment (Figure 3.5). The significance of such enhancement with DS, is firstly that it would increase sensitivity in detection of C-proteinase in chromatographic samples, and secondly it could be used to increase the quantity of cleaved C-propeptide to confirm the cleavage sites of human C-proteinase (Section 3.5.2). From these results it was decided to use 0.02% (w/v) DS in the enhanced electrophoretic assay throughout the study.

3.3 PURIFICATION OF CHICK C-PROTEINASE

3.3.1 Background to the Purification Procedures

A number of sources of C-proteinase previously investigated showed that chick tendon from 17 day old embryos had the most abundant levels. However, according to the purification procedures developed by Hojima (Hojima *et al.*, 1985) the low levels of enzyme expressed in tendon cultures (<1 μg/g tendon) meant that organ culture media from at least 300 doz embryos would be required for each round of purification (to obtain ~40 μg enzyme). Prior to initiating this project, the procedure in the laboratory was to dissect 25 doz embryos/week, and to culture them in 500 ml of media. At this rate it would take three months to generate enough media for one round of purification.

Figure 3.5 Effect of 0.02% (w/v) DS on Human C-proteinase Cleavage of Procollagen



Human procollagen was cleaved with partially purified human C-proteinase assayed and analysed by SDS-PAGE (7% separating), under reducing conditions, and separated proteins detected by fluorography. ***Lane 1***, E-A (0.3 units) added to 1 μg human procollagen and incubated for 4 hr at 37 °C. ***Lane 2***, as in lane 1 with 0.02% (w/v) DS. Complete cleavage is seen, the pNα1(I) band runs close to where the proα1(I) was prior to cleavage.

The first three purifications using this dissection protocol, which all gave low yields of active C-proteinase (Table 3.1), where used as a foundation to modify the culturing technique in order to purify active chick C-proteinase (purifications 4 and 5).

The first purification (Table 3.1, puri. 1) from 3.75 L of cultured media yielded only 20-50 units of C-proteinase activity which had a specific activity of 0.02 U/mg. It appeared that activity and protein was lost on the concanavalin-A and heparin-Sepharose columns. It was decided that 5 L of starting media would be required to elute >50 mg of protein from the Green-A column. In the second purification (Table 3.1, puri. 2) to maximise activity of C-proteinase (a calcium dependent enzyme) solid $CaCl_2$ (5 mM) was added to the starting media (5 L). However, a white precipitate immediately formed, which was collected, and the purification was continued using the supernatant. Only 7.9 mg of protein was eluted from the Green A column, and less than 1 mg of protein was washed through the heparin-Sepharose column, which subsequently had no activity. In order to determine whether C-proteinase was present in the precipitate it was resuspended in storage buffer, dialysed into Green A equilibration buffer and loaded on to this first affinity column. However, no activity was detected in samples taken from eluted fractions from the Green A DyeMatrex column and the purification was stopped.

To speed the generation of cultured media, the number of embryos dissected was doubled to 50 doz, the volume of culturing DMEM increased to 1 L, and 5% CO_2 and 95% air bubbled through to ensure the larger number of tendons (15-30g compared to

Table 3-1 Summary of Preliminary Chick C-proteinase Purifications

Puri. (No.)	Media (L)	Embryo (doz)	Tendon (g)	†Time (mth)	Green (mg)	Con A (mg)	H/S E-A (mg)	Activity (U/mg)	H/S E-B (mg)	Activity (U/mg)	S300/E-A (U/mg)	S300/E-B
*Puri 1	3.75	225	82	2	50.7	7.2	3	/	1	/	<1 mg	0
*Puri 2	5	300	110	3	7.9	2.6	<1	n.d			0.75	
[1]*Puri 3	3.8	225	85	1	52	4.3	1.9	n.d	<1	0		

Puri.- purification /**Con-A**- concanavalin A-Sepharose /**H/S**- heparin-Sepharose /**S300**- Sephacryl 300
†Time taken to dissect embryos
*Purifications using old dissection and culturing conditions
[1]*Purification after scaling up dissection (50 doz chicks/week)

12-20g with 25 doz) had optimum growth conditions. After taking one month to obtain 3.8 L of media from 225 doz embryos another purification was carried out (Table 3.1, puri. 3). The eluted protein from the Green A column was 52 mg, a 2.5% increase compared to purification 1, which is explained by the 3% increase in tendon yield. However, only 4.3 mg of protein (8.2% of load) was recovered from the concanavalin A-Sepharose column, this was not enough to purify further any active enzyme from the heparin-Sepharose column.

A major problem with scaling up dissection was that by the end of the second day of culturing, over 50% of cultures were contaminated. These were not pooled with day one cultures, but possible contaminants (such as bacteria and proteases) could have been present in these earlier cultures, and may have affected enzyme activity. To reduce risk of contamination, whilst ensuring sufficient nutrients were available, the tendons from 50 doz embryos were divided in half, and cultured separately in 500 ml DMEM. With this new protocol <10% of cultures were contaminated by the third day of culture. Two successful purifications followed, using this new culturing protocol (purifications 4 and 5). Active E-A and E-B forms of C-proteinase were partially purified in both purification 4 and 5, and the purification stages of purification 4 are described in detail below.

3.3.2 Chick C-proteinase, Purification 4: Affinity Chromatography

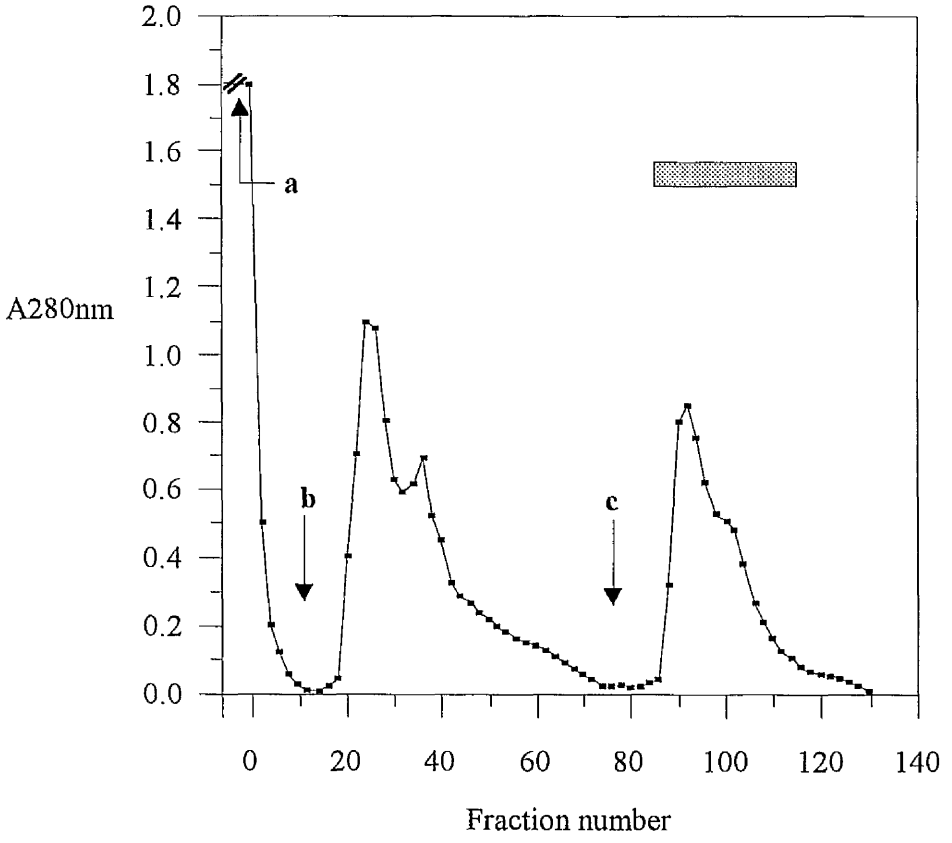
3.3.2.1 Green A DyeMatrex gel chromatography

The gel, composed of a triazinyl dye immobilised on cross-linked agarose, has reversible affinity for C-proteinase and is used to separate active enzyme from contaminant proteins (~95% of load). Organ culture media from tendons dissected from 400 doz chick embryos was applied to the column (Figure 3.6). Typically in all purifications ~85% of applied protein was washed through the column, and ~10% eluted by equilibration buffer (0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl) as the first well defined peak. Samples from this eluted peak analysed by electrophoretic assay were inactive. Active C-proteinase, bound tightly to the resin was eluted by elution buffer (containing 3 M NaCl and 2 M urea), and the 310 ml pool (120 mg protein) concentrated to 62 ml (111 mg protein) by pressure ultrafiltration (1.8 mg/ml, 91% protein recovery on concentration).

3.3.2.2 Concanavalin A-Sepharose chromatography

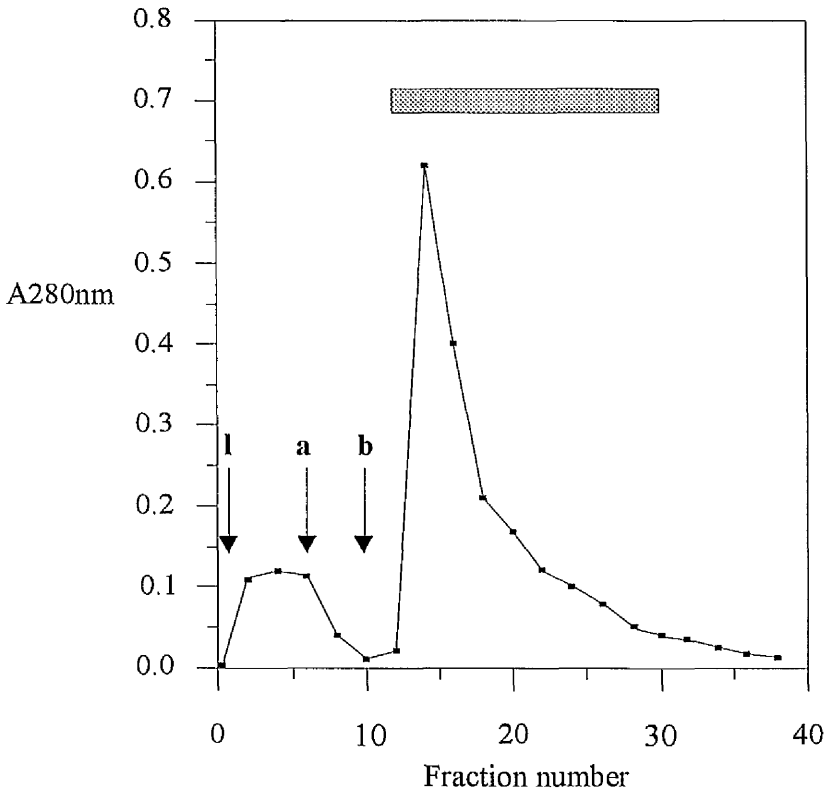
The lectin concanavalin A, immobilised to Sepharose 4B, was used as the second column. The active sample from Green A DyeMatrex chromatography diluted with an equal volume of 0.15 M Tris-HCl buffer, pH 7.5, containing 2 mM $CaCl_2$, was applied to a column of concanavalin A-Sepharose (Figure 3.7). Equilibration buffer applied to the column eluted proteins (5 mg, 4.5% of the load) not adsorbed to the column. The second protein peak eluted with buffer including 0.7 M α-methyl-D-mannoside (which competes for the carbohydrate), contained active C-proteinase and was pooled (290 ml, 26 mg, 23% of the load). During pressure ultrafiltration (to 35 ml) with repeated

Figure 3.6 Green A DyeMatrex Chromatography of Chick C-proteinase



Culture medium (7.7 L), made up to the buffer concentrations of the Green A DyeMatrex equilibration buffer, was applied to a Green A column (5cm x 10cm) at a flow rate of 130 ml/hr and eluted as follows: **a,** equilibration buffer at the end point of loading culture medium; **b**, elution buffer 1, with 1 M NaCl; **c**, elution buffer 2, with 2 M NaCl and 3 M urea. The elution was monitored by measuring the absorbance (A280nm) of the 10 ml fractions. Fractions (85-115, shaded box) containing active C-proteinase were pooled.

Figure 3.7 Concanavalin A-Sepharose Chromatography of Chick C-proteinase



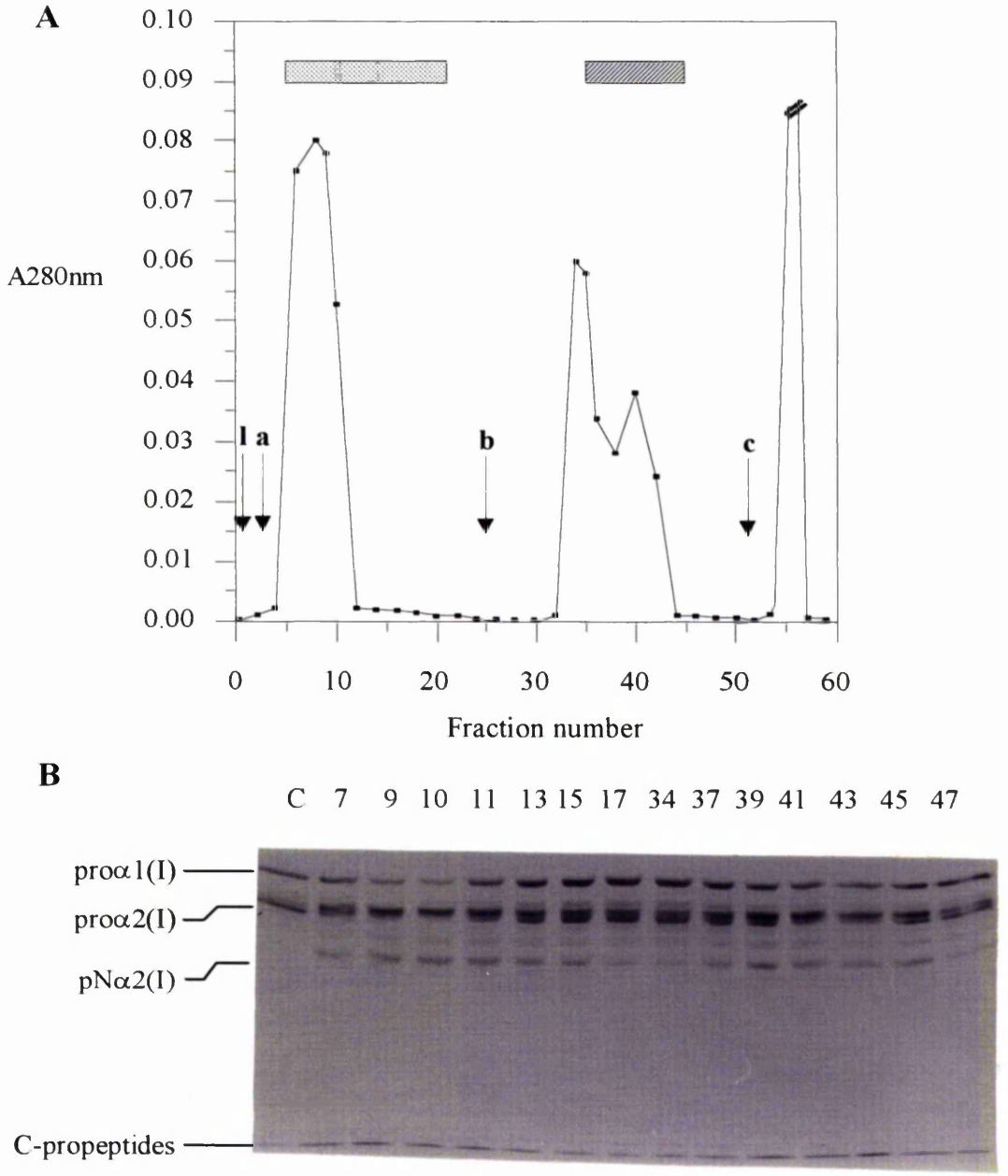
Pooled fractions from the Green A chromatography were diluted to the same buffer concentrations as the concanavalin A-Sepharose buffer and applied to a concanavalin A-Sepharose column (1.5cm x 11cm) at a flow rate of 25 ml/hr. After loading (*l*) the column was eluted by applying **a**, equilibration buffer and **b**, elution buffer (at 5 ml/hr). The absorbance at 280nm was determined for alternate 10 ml fractions. Fractions containing C-proteinase activity were pooled (12-30, shaded box).

washings of the membrane with α-methyl-D-mannoside, only 13 mg of protein was recovered. Typically, in all chick purifications only 20-30% of protein was removed from the column. Attempts in eluting the remaining tightly bound proteins using 1 M α-methyl-D-mannoside, 2 M NaCl and 1.5 M urea concentrations failed.

3.3.2.3 Heparin-Sepharose chromatography

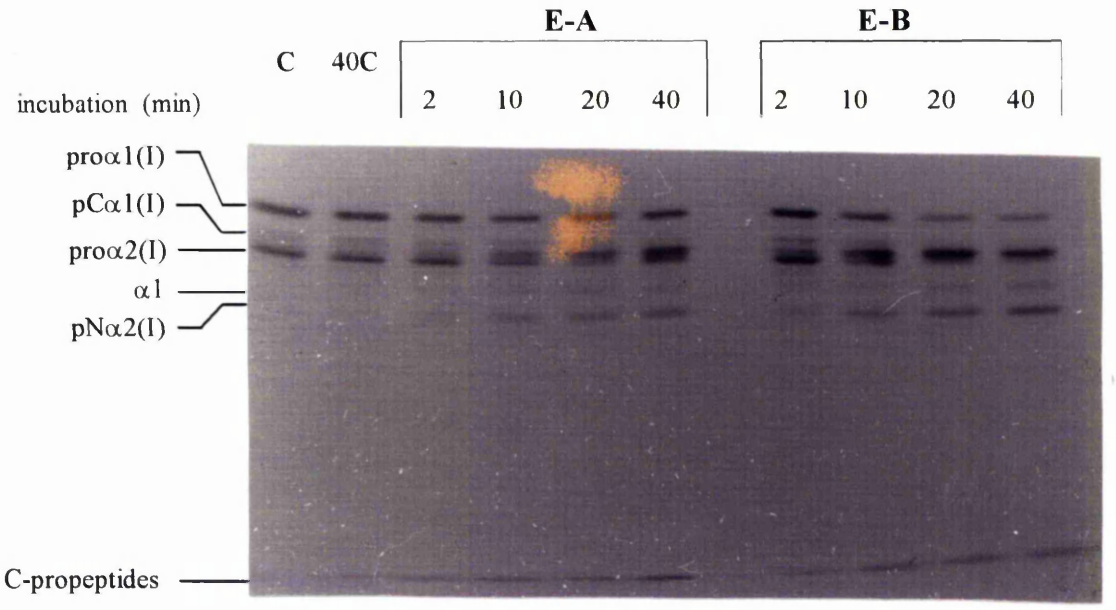
Heparin, from porcine intestinal mucosa, is a highly sulphated glycosaminoglycan and when conjugated to Sepharose CL-6B binds to a wide range of biomolecules, including C-proteinase. The concentrated sample from concanavalin A-Sepharose chromatography was dialysed against 0.3 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl and 0.005% (w/v) Brij-35 (equilibration buffer), was centrifuged and the supernatant applied to the heparin-Sepharose column (Figure 3.8, A). Equilibration buffer (350 ml) applied after the load, eluted a first peak of unbound protein, and 0.3 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.005% (w/v) Brij-35 (400 ml) eluted a second peak. Cleaning buffer, 0.3 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, 1 M urea and 0.005% (w/v) Brij-35 (200 ml) eluted tightly bound proteins (20% of total). Samples from fractions were analysed by the basic electrophoretic assay (Figure 3.8, B), and C-proteinase was pooled as indicated. Heparin-Sepharose chromatography separated C-proteinase activity into two peaks, bound E-B and unbound E-A. The first pool, E-A, (170 ml, 5.2 mg) was concentrated to 8 ml (4 mg), and the second, E-B, (110 ml, 5 mg) concentrated to 8.5 ml (3.4 mg), by pressure ultrafiltration. C-proteinase activity, in the two batches, was determined by assaying 5 μl samples using the basic electrophoretic assay (Figure 3.9). From the autoradiograph

Figure 3.8 Heparin-Sepharose Chromatography of Chick C-proteinase



A, Pooled fractions from concanavalin A-Sepharose chromatography were dialysed against equilibration buffer and applied to a heparin-Sepharose column (2.5cm x 10cm) at a flow rate of 25 ml/hr (**l**) and collected as 10 ml fractions. Proteins were eluted as follows: **a**, equilibration buffer; **b**, elution buffer; **c**, cleaning buffer. ***B,***. Samples (5 µl) from fractions (numbers as indicated) were added to 1 µg CET-procollagen, incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. Two active peaks were pooled: fractions 5-21 (shaded box) from the wash, named E-A, and fractions 35-45 (hatched box) from the elution, named E-B.

Figure 3.9 Activity of Purified Chick E-A and E-B from Heparin-Sepharose Chromatography



Chick C-proteinase was assayed by electrophoretic assay. C-proteinase samples (5 µl) from E-A and E-B were incubated with 1 µg CET-procollagen, for 2, 10, 20 and 40 min at 37 °C, and analysed by SDS-PAGE (7% separating), under reducing conditions, and separated proteins detected by fluorography. ***Lane C,*** 1 µg CET-procollagen unincubated; ***lane 40C***, 1 µg CET-procollagen incubated for 40 min.

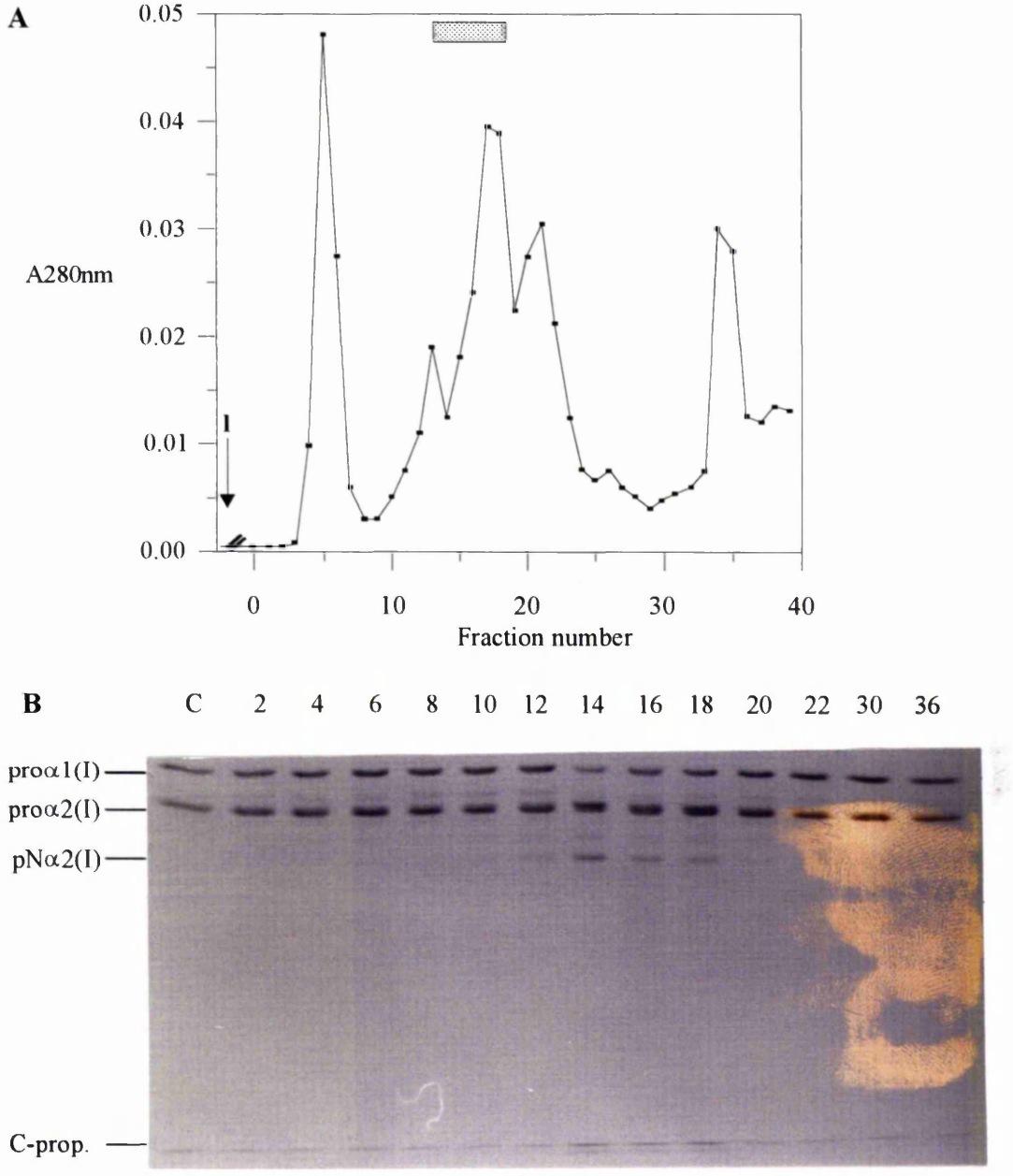
of the fluorogram the % cleavage was calculated (equation 1, section 2.5), and the initial rate of cleavage determined by plotting % cleavage against time of incubation. E-A contained 625 units/ml (5000 total units purified, 1250 units/mg), and E-B 720 units/ml (6120 total units, 1800 units/mg). According to Hojima *et al.*, (Hojima *et al.*, 1985) the specific activity of pure C-proteinase is 30 000 units/mg, from this the purity of E-A was estimated to be 4.2% (170 μg C-proteinase), and E-B 6% (200 μg C-proteinase).

Samples of E-A and E-B were radiolabelled with ^{14}C-formaldehyde, and 10 μl samples analysed by SDS-PAGE (7% separating gel) under reducing conditions, and fluorography (result not shown). At least 30 and 25 bands were present in the E-A and E-B samples, respectively. C-proteinase from this fourth purification was designated for studies to obtain a N-terminal sequence (Section 3.3.3.1).

3.3.3 Sephacryl-300 Gel Filtration of E-A from Purification 5

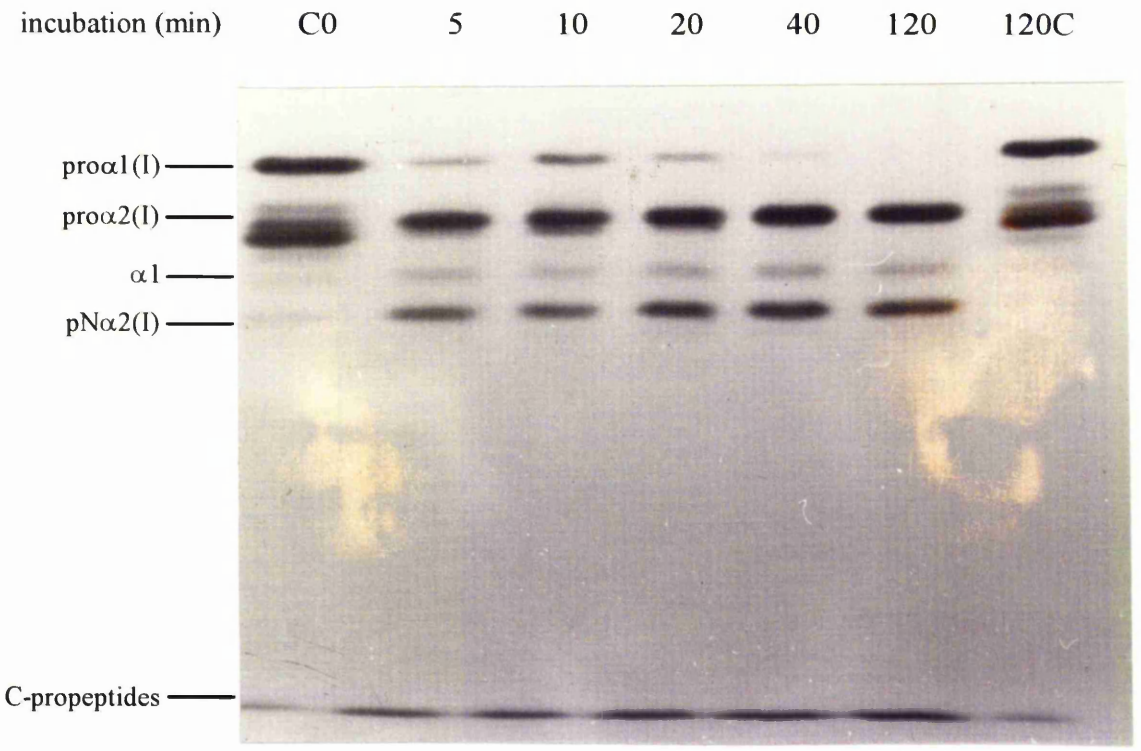
E-A C-proteinase eluted from the heparin-Sepharose column in purification 5 (Figure 3.13, B), was purified by gel filtration using a Sephacryl-300 column. The sample was dialysed against S-300 equilibration buffer and loaded on to the column (Figure 3.10). Eluted enzyme was monitored by assaying fractions using the basic electrophoretic assay (Figure 3.10, B), active fractions were pooled (60 ml) and concentrated to 3 ml (0.9 mg) by pressure ultrafiltration. The C-proteinase activity was 740 units/ml (total 2220 units, 2500 units/mg) (Figure 3.11). Gel filtration resulted in a 1.8 fold in

Figure 3.10 Sephacryl-300 Gel Filtration of Chick C-proteinase (E-A, purification 5)



A, Pooled fractions from heparin-Sepharose chromatography were dialysed into S-300 equilibration buffer and applied to a S-300 gel filtration column (2.5cm x 110cm) at a flow rate of 15 ml/hr (**I**). The absorbance was determined for alternate 10 ml fractions. *B*, Samples (10 µl) from alternate fractions were added to 1 µg CET-procollagen, incubated for 4 hr at 37 °C and analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. Active fractions (13-18, shaded box) were pooled. C-prop: C-propeptides

Figure 3.11 Activity of Purified Chick E-A from S-300 Gel Filtration



Chick C-proteinase samples (5 µl) from concentrated E-A (Figure 3.10) were incubated with 1 µg CET-procollagen, for 5, 10, 20, 40, and 120 min at 37 °C, and analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. ***Lane C0***, 1 µg CET-procollagen unincubated; ***lane 120C***, 1 µg CET-procollagen incubated for 120 min.

purification, the pooled enzyme was 8.3% pure compared to 4% after the heparin-Sepharose column. C-proteinase purified from this fifth purification was used in fibril formation studies.

3.3.3.1 Sequence analysis of chick C-proteinase from purification 4

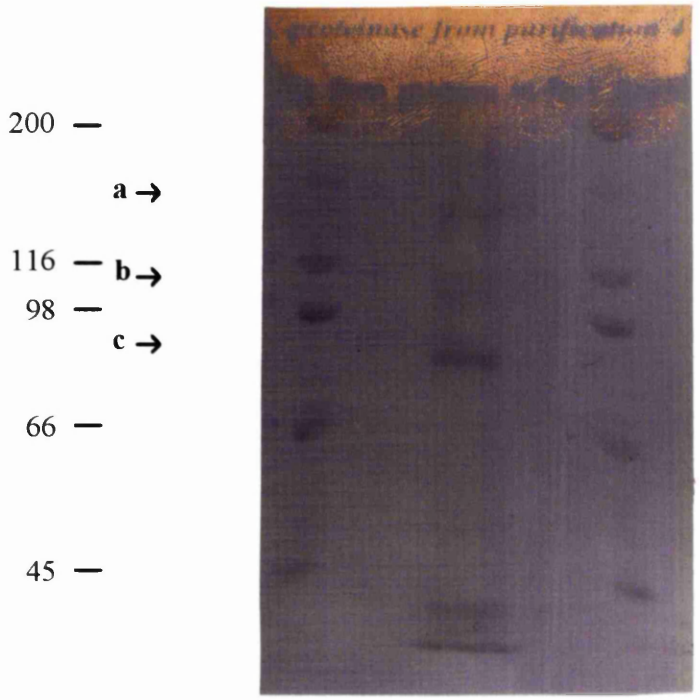
In order to obtain N-terminal sequences from proteins in E-A (purification 4), 1 ml (0.5 mg protein) was concentrated to 100 µl, separated by SDS-PAGE, and blotted to PVDF membrane (Figure 3.12, A). Three bands were apparent in the correct molecular weight range, were subjected to N-terminal sequencing. However, only the 95.5 kDa band gave a signal during N-terminal sequence analysis (Figure 3.12, B). The sequence shown is incomplete, as the signal was weak, either because of low sample yield or because the N-terminus was blocked.

3.3.3.2 Synopsis of purifying chick C-proteinase

Using an established protocol devised by Hojima *et al.*, two active forms of C-proteinase, E-A and E-B were partially purified from leg tendons from ~400 doz chick embryos. However, one of the main disadvantages of using chick tendon, as the source of C-proteinase, was the dissection time and the increase in contamination of organ cultures associated with scaling up dissection to 50 doz/week. After these problems were eradicated, active enzyme was successfully purified with ~5000 units of activity. Summaries of these purifications (number 4 and 5) are shown in figure 3.13. However both forms of enzyme, E-A and E-B, were only 4-6% pure, with specific activities of 1250-1800 units/mg.

Figure 3.12 N-Terminal Sequence Analysis of Electrophoretically Purified Chick E-A C-proteinase

A



B

a: weak signal-no sequence obtained
b: weak signal-no sequence obtained

c: Gln-Pro-Glu-Pro-Ile-Thr----

A, C-proteinase E-A sample (purification 4) was concentrated by freeze drying and reconstituted in 1 x sample buffer and subjected to SDS-PAGE (7% separating), under reducing conditions. The separated proteins on the polymerised gel were blotted to PVDF using a 10 mM CAPS, pH 11 and 10% (v/v) methanol transfer buffer. Protein bands, indicated by letters **a** (151 kDa), **b** (108 kDa) and **c** (95.5 kDa), were excised and sent for N-terminal sequencing. *B*, Amino-acid signals from **a** and **b** were weak and no N-terminal sequence was obtained. Six residues were obtained from **c**.

Figure 3.13 Summaries of Chick C-proteinase Purifications 4 and 5

A, Purification 4

	Volume (ml)	Protein (mg)	Units of activity	Recovery (%)	Units/mg	Fold
Crude media	7750	2100	10100	100	4.8	1
Green A	62	111	9250	92	83.3	17
Concanavalin A	35	13	7800	78	600	125
Heparin Sepharose E-A	8	4	5000	50	1250	260
Heparin Sepharose E-B	8.5	3.4	6120	61	1800	375

B, Purification 5

	Volume (ml)	Protein (mg)	Units of activity	Recovery (%)	Units/mg	Fold
Crude media	6400	1990	12500	100	6.28	1
Green A	55	63.6	10375	83	163	26
Concanavalin A	30	7.3	7060	56	967	154
Heparin Sepharose E-A	6	2.7	5225	42	1935	308
Heparin Sepharose E-B	5.5	1.5	400	3.2	266	42
S300 E-A	3	1	2200	18	2200	350

Protein (mg) was determined by the absorbance at 280nm
Activity was determined using the basic electrophoretic assay, where samples were analysed by SDS-PAGE and units calculated by plotting % cleavage of proα1(2) against time
Recovery is % recovery of activity units after concentrating the pool, relative to the activity obtained from previous column
Fold purification was determined by comparing specific activities (units/mg)

With the yield of enzyme as low as 0.4 μg/g of tendon not enough C-proteinase was available to raise antibodies, and with purity estimated to be only 4-6% the active band could not be determined. A six residue N-terminal amino acid sequence was obtained from a 95.5 kDa band, however the signal was weak and it was presumed that cyclisation of the N-terminal Gln residue had occurred. The short sequence obtained was not suitable for the generation of cDNA primers due to the redundancy inherent in its sequence, and so a cloning approach to characterising C-proteinase was not attempted.

For these reasons an alternative source of enzyme was sought, and a number of cell lines were examined. MG63 human osteosarcoma cells had been shown to have a 40% increased synthesis of types I and III procollagen compared to cultured skin fibroblasts (Jukkola *et al.*, 1993), and thus could reasonably be expected to have a high expression of C-proteinase. MG63 is an established cell line, and as the cells grow well in culture, they can be readily maintained for bulk harvesting of media. This novel source of enzyme was used to partially purify and to characterise, human C-proteinase for the first time, as is described in the following section.

3.4 PURIFICATION OF HUMAN C-PROTEINASE FROM MG63 CELLS

3.4.1 Cell Lines

Five cell lines were examined for their active production of C-proteinase into growth media over a 3 day incubation. Cells, seeded at 1.5 x 10^5 in 6 well plates were washed after 24 hr in 2 x 2 ml of Nutridoma supplemented DMEM complete media and left for

4 hr. This media was replaced with 1 ml of fresh media, and the cells incubated at 37 °C for 3 days. Every 24 hr, 89µl of media was assayed using the enhanced electrophoretic assay, and enzyme activity determined from the autoradiograph of the fluorogram (Table 3.2). The three cancer lines HOS, HT1080 and MG63 synthesised 2 fold more active enzyme compared to the fibroblasts (C6B5 and MRC5).

In a separate experiment SAOS-2 cells were also analysed for their ability to produce C-proteinase. This cell line produced approximately 2/3 fold more C-proteinase compared to the MG63 cells, however, there also was a significant amount of N-proteinase present. MG63 is an established cell line that secretes steady amounts of C-proteinase, which increase with time, into the crude media (Table 3.2). Media from these MG63 cells also contained less interfering N-proteinase, as seen in SAOS-2 cells, and for these reasons it was decided to use MG63 as the human C-proteinase source.

3.4.2 Preliminary Investigation to Optimise Crude Culture Conditions

MG63 cells were expanded into four 175 cm^2 flasks each containing 1 x 10^6 cells/flask. The cells were cultured in 25 ml DMEM containing 50 µg/ml ascorbate to enhance deposition of a pericellular matrix. At 72 hr the cells were 90% confluent, and after 5 days incubation at 37 °C the cells were mutilayered but still viable. The media was then removed and cells fed with a further 25 ml/flask. After the second harvest, 89 µl samples of media from the combined harvest of 200 ml were assayed by the

Table 3-2 C-proteinase Activity in Cell Lines

	% cleavage		
	24 (hr)	48 (hr)	72 (hr)
C6B5 adrenal fibroblast	17	16	27
HOS osteosarcoma	35	34	33
HT1080 fibrosarcoma	28	31	33
MRC5 lung fibroblast	10	16	17
MG63 osteosarcoma	28	35	38

Culture media (89µl) from 5 different cell lines grown in 1 ml of DMEM medium for the above incubation times was added to 1 µg CET-procollagen, with 0.02% (w/v) DS, in correct buffer conditions and incubated for 4 hr. Samples were analysed by SDS-PAGE under reducing conditions, and separated proteins detected by fluorography. The % cleavage was determined from densitometry of protein bands. The data is from a one sample experiment.

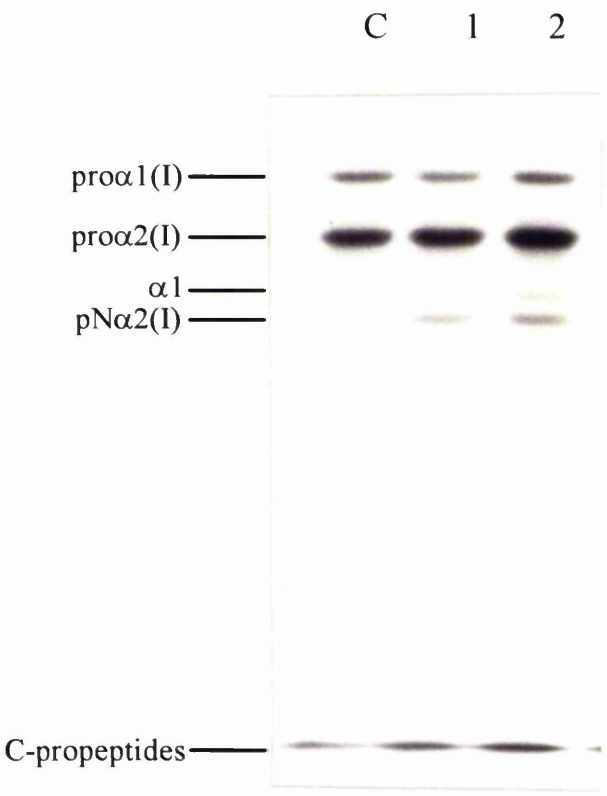
SDS-PAGE methods. The results showed that the concentration of C-proteinase in the culture medium was 1.6 unit/ml (Figure 3.14).

To determine how long the cells could be cultured for without loss in production of C-proteinase, MG63 cells were cultured for 4 weeks. Media (89 μl) from the MG63 cells growing in DMEM complete media was removed every day over the 4 weeks and assayed using the SDS-PAGE assay (media was replenished every four days). Detectable C-proteinase was seen after 3 days and after replenishment the levels of enzyme activity were restored after 24 hr. The level of enzyme activity remained constant after 10 days. This protocol was used to generate starting media to obtain partially purified human C-proteinase for the first time, as described in detail below.

3.4.3 Preparation of Crude Starting Media

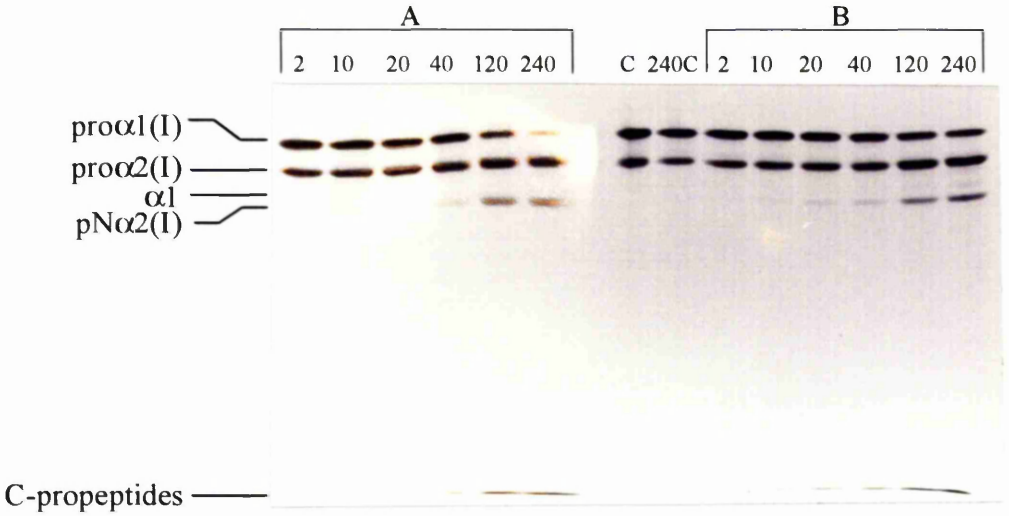
A total of 6.6 L of media was harvested by collecting every 4 days from cultured MG63 cells (500 ml/harvest). Each round of harvest assayed for C-proteinase was active, and was combined in a final pool which was also assayed using the enhanced SDS-PAGE method (Figure 3.15, A). Final Tris-HCl, NaCl and NaN_3 concentrations in the sample were made up to 0.05 M, 0.3 M and 0.02% (w/v) respectively and the pH adjusted to 8.1, with a loss of 2% of activity (Figure 3.15, B). Activity, was calculated from the initial rate of proα2(I) cleavage determined from the intensities of bands using equation 1 (Section 2.5). Total activity was determined as 13600 units, in the presence of DS, with an estimated actual activity of 6800. The total protein (1452 mg) in the media was determined by absorbance at A280nm, which gave a specific

Figure 3.14 C-proteinase Activity in Media from MG63 Cells



MG63 cells were grown in four 175 cm^2 flasks with 25 ml/flask of DMEM complete medium for 5 days. The media was harvested and replaced with fresh media for a further 5 days. Samples were assayed using the basic assay, and analysed by SDS-PAGE (7% separating) under reducing conditions, and proteins detected by fluorography. ***Lane C***, 1 μg CET-procollagen incubated for 2 hr at 37 °C. ***Lane 1***, 1 μg CET-procollagen incubated with 1 unit of chick E-A C-proteinase for 2 hr. ***Lane 2***, 89 μl of media from the combined harvests (200 ml) from MG63 cells incubated with 1 μg CET-procollagen for 2 hr.

Figure 3.15 C-proteinase Activity in Pooled Media from Human MG63 Cells



Samples (45 µl) from cultured media from MG63 cells were incubated with 1 µg CET-procollagen, in the presence of 0.02% (w/v) DS, for 2, 10, 20, 40, 120 and 240 min at 37 °C, and analysed by SDS-PAGE (7% separating) under reducing conditions, and proteins detected by flurography. ***A***, crude media; ***B***, crude media after the addition of Tris-HCl, NaCl, $CaCl_2$ and adjusting the pH to 8. ***Lane C***, 1µg CET-procollagen unincubated; ***lane 240C***, 1 µg CET-procollagen incubated for 240 min.

activity of 4.68 units/mg.

3.4.4 Affinity Chromatography

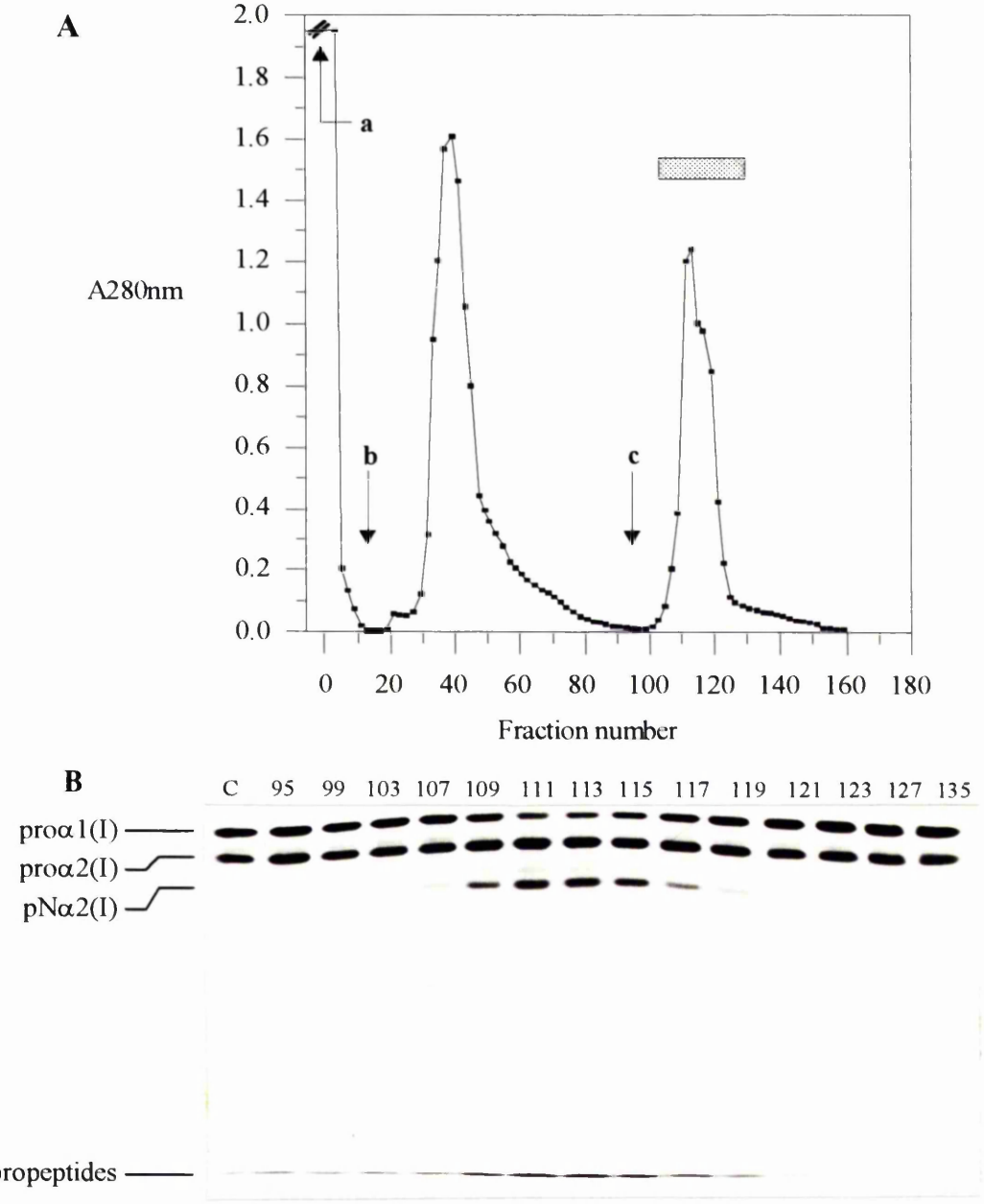
3.4.4.1 Green A DyeMatrex chromatography

The MG63 media (6.6 L) was centrifuged to remove cell debris and the supernatant applied to the Green A dyeMatrex column (Figure 3.16, A). Approximately 80% of protein applied was washed through, 14.5% eluted with equilibration buffer and 6.5% eluted as the second peak with 0.05 M Tris-HCl buffer, pH 7.5, containing 3 M NaCl and 2 M urea. Active fractions determined by the enhanced electrophoretic assay (Figure 3.16,B) were pooled and concentrated from 280 ml to 62.5 by pressure ultrafiltration, with 65% of protein, and 90% of total activity (5720 units) recovered. This first stage gave a 19.6 fold purification of the enzyme, with 84% of the original activity recovered.

3.4.4.2 Concanavalin A-Sepharose chromatography

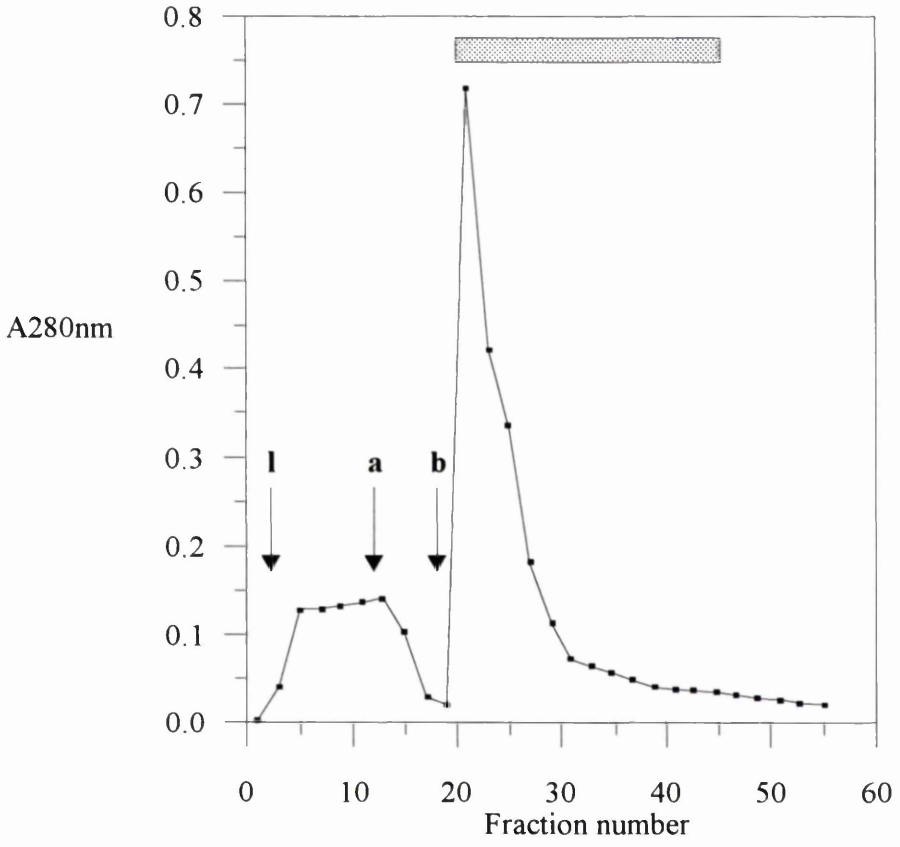
The 62.5 ml concentrated sample from Green A DyeMatrex chromatography was diluted with 62.5 ml of 0.15 M Tris-HCl buffer, pH 7.5, containing 2 mM $CaCl_2$, and applied to the column (Figure 3.17). Equilibration buffer (180 ml) washed through 15 mg of protein (24% of load) not adsorbed to the column, and 55% of the load was eluted with 360 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.7 M α-methyl-D-mannoside, 1.5 M NaCl and 1 M urea. The recovery of total protein from the column

Figure 3.16 Green A DyeMatrex Chromatography of Human C-proteinase



A, Medium (6.6 L) made up to the buffer concentrations of the Green A equilibration buffer was applied to a Green A column (5cm x 10cm) at a flow rate of 130 ml/hr and eluted as follows: ***a***, equilibration buffer at the end point of loading culture medium; ***b***, elution buffer 1, with 1 M NaCl; c, elution buffer 2, with 2 M NaCl and 3 M urea. The elution was monitored by measuring the absorbance (A280nm) of the 10 ml fractions. Samples (5 µl) from alternate fractions were added to 1 µg CET-procollagen, incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. ***B, lane C***, 1 µg procollagen incubated without enzyme. The presence of pNα2(I) indicates C-proteinase activity. Fractions 101-129 (shaded box) were pooled.

Figure 3.17 Concanavalin A-Sepharose Chromatography of Human C-proteinase



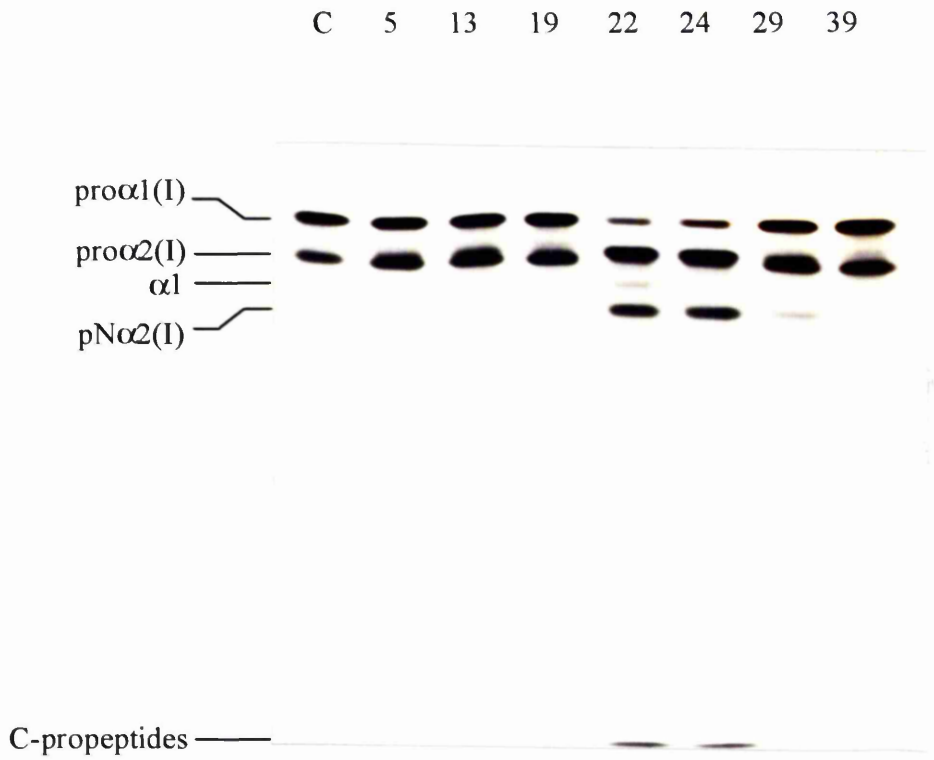
Pooled fractions from the Green A chromatography were diluted to the same buffer concentrations as the concanavalin A-Sepharose buffer and applied to a concanavalin A-Sepharose column (1.5cm x 11cm) at a flow rate of 25 ml/hr. After loading (*l*) the column was eluted by applying **a**, equilibration buffer; **b**, elution buffer (at 5 ml/hr). The absorbance at 280nm was determined for alternate 10 ml fractions. Fractions were assayed to locate active C-proteinase (Figure 3.18).

was greater than in the chick purification where only 27.5% of protein was recovered. To locate active C-proteinase, 5 µl samples were assayed (Figure 3.18), and fractions containing enzyme were pooled (31 mg of protein). The 255 ml pool was concentrated by pressure ultrafiltration to 30 ml, with three washes of elution buffer, and 29 mg of protein finally recovered. Activity of the concentrated sample determined by the basic electrophoretic assay, was 96 units/ml, a total of 2880 units (a 50.3% recovery of activity). A 1.1 fold purification of the enzyme was achieved with the concanavalin A-Sepharose column.

3.4.4.3 Heparin-Sepharose chromatography

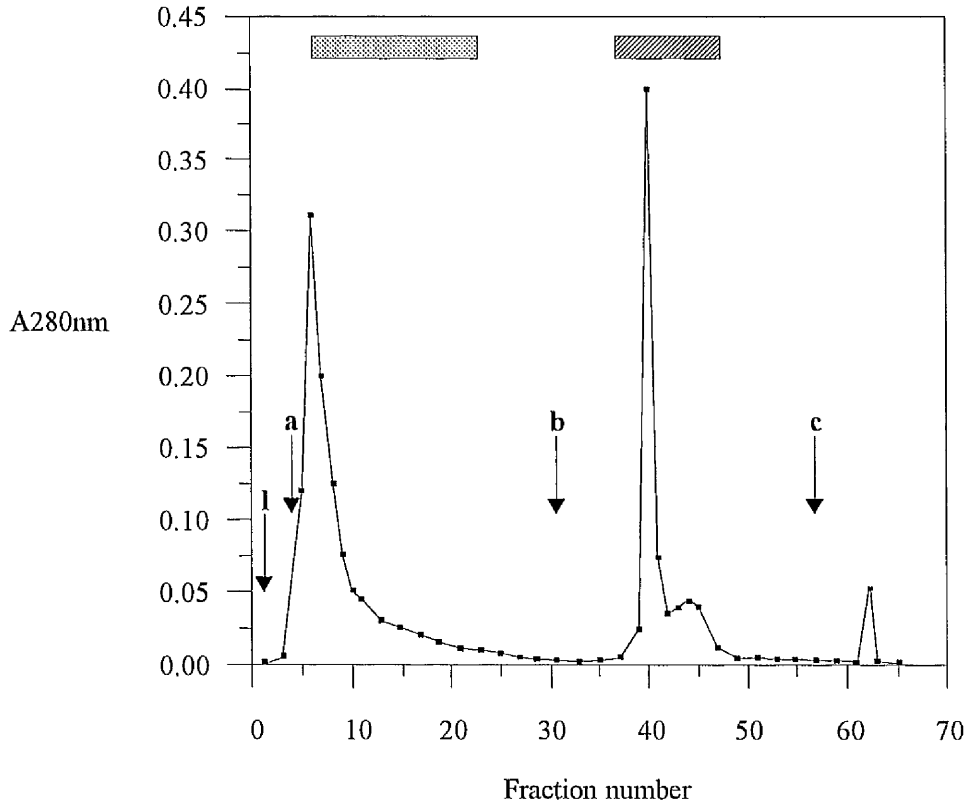
The concanavalin A-Sepharose sample dialysed against 0.3 M Tris-HCl buffer, pH 7.5, containing 0.05 M NaCl and 0.005% (w/v) Brij-35 (equilibration buffer), was centrifuged, and the supernatant (34 ml) loaded on to the column (Figure 3.19). Equilibration buffer (310 ml) eluted the first peak of unbound protein, and 0.3 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl and 0.005% (w/v) Brij-35 (260 ml) eluted the second protein peak. Cleaning buffer, 0.3 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, 1 M urea and 0.005% (w/v) Brij-35 (200 ml) eluted a further 4% of protein bound to the column. Samples from fractions were assayed using the enhanced electrophoretic assay (Figure 3.20). Activity was located in the first two protein peaks, and fractions were pooled accordingly. The first pool (180 ml), named E-A containing 14.4 mg of protein (50% of load) was concentrated to 5.5 ml, after adding NaCl, urea and $CaCl_2$ to concentrations of 1 M, 1 M and 5 mM respectively. Approximately 36% of protein in the peak was not recovered after pressure

Figure 3.18 Electrophoretic Assay of Concanavalin A-Sepharose Fractions



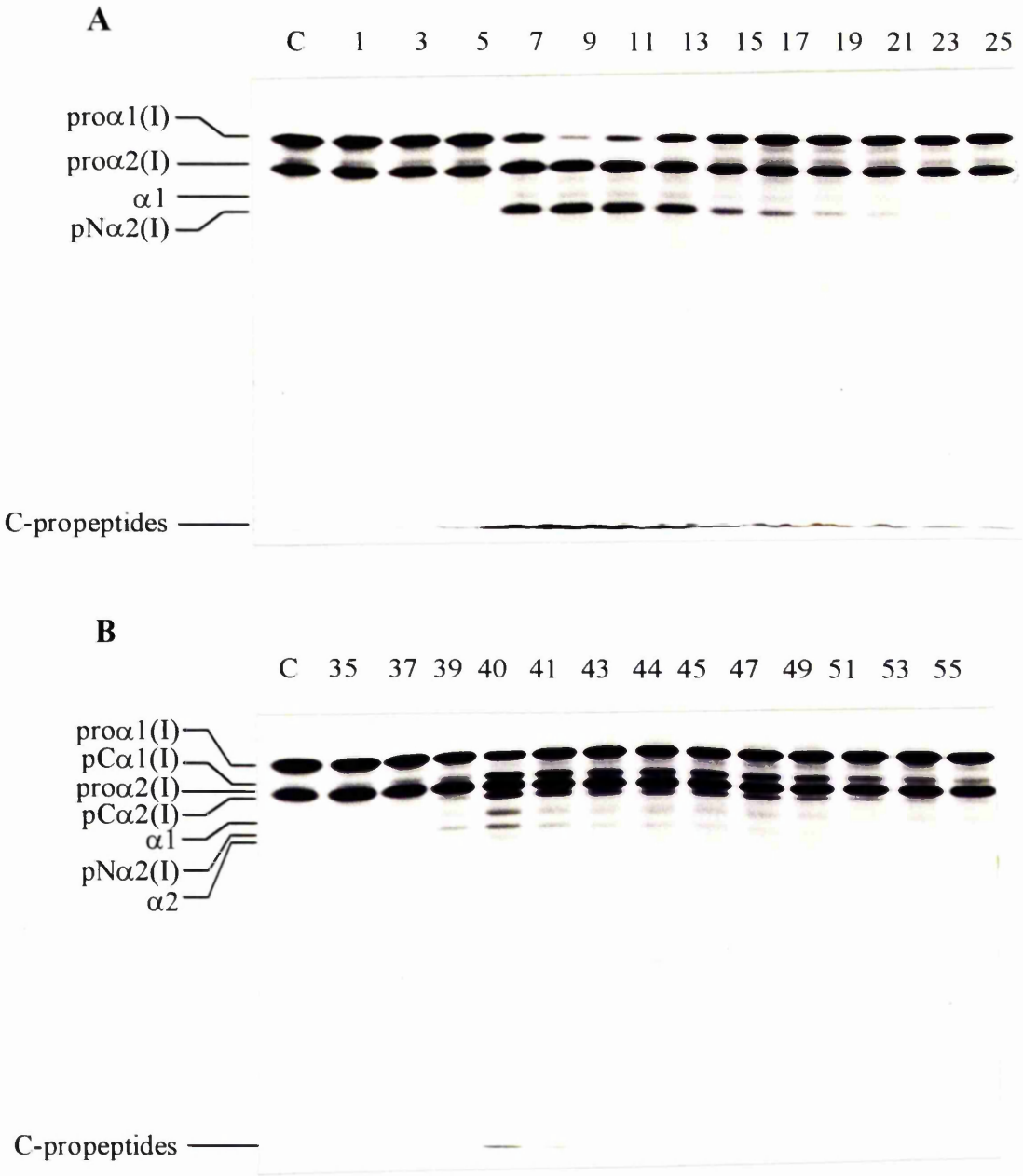
Fractions relate to figure 3.17. Chick C-proteinase was assayed by electrophoretic assay. C-proteinase samples (5 µl) from E-A and E-B were added to 1 µg CET-procollagen, (with 0.02% (w/v) DS), incubated for 4 hr at 37 °C, and analysed by SDS-PAGE (7% separating), under reducing conditions, and separated proteins detected by fluorography. ***Lane C***, 1 µg procollagen incubated without enzyme. Fractions (20-45, shaded box) containing active enzyme were pooled.

Figure 3.19 Heparin-Sepharose Chromatography of Human C-proteinase



Pooled fractions from concanavalin A-Sepharose chromatography were dialysed against equilibration buffer and applied to a heparin-Sepharose column (2.5cm x 10cm) at a flow rate of 25 ml/hr (**l**) and collected as 10 ml fractions. Proteins were eluted as follows: **a**, equilibration buffer; **b**, elution buffer; **c**, cleaning buffer. Two active enzyme peaks were detected by electrophoretic assay (Figure 3.20), pooled and named E-A (fractions 6-23, shaded box) and E-B (fractions 37-47, hatched box).

Figure 3.20 C-proteinase Assay of Samples from Heparin-Sepharose Chromatography



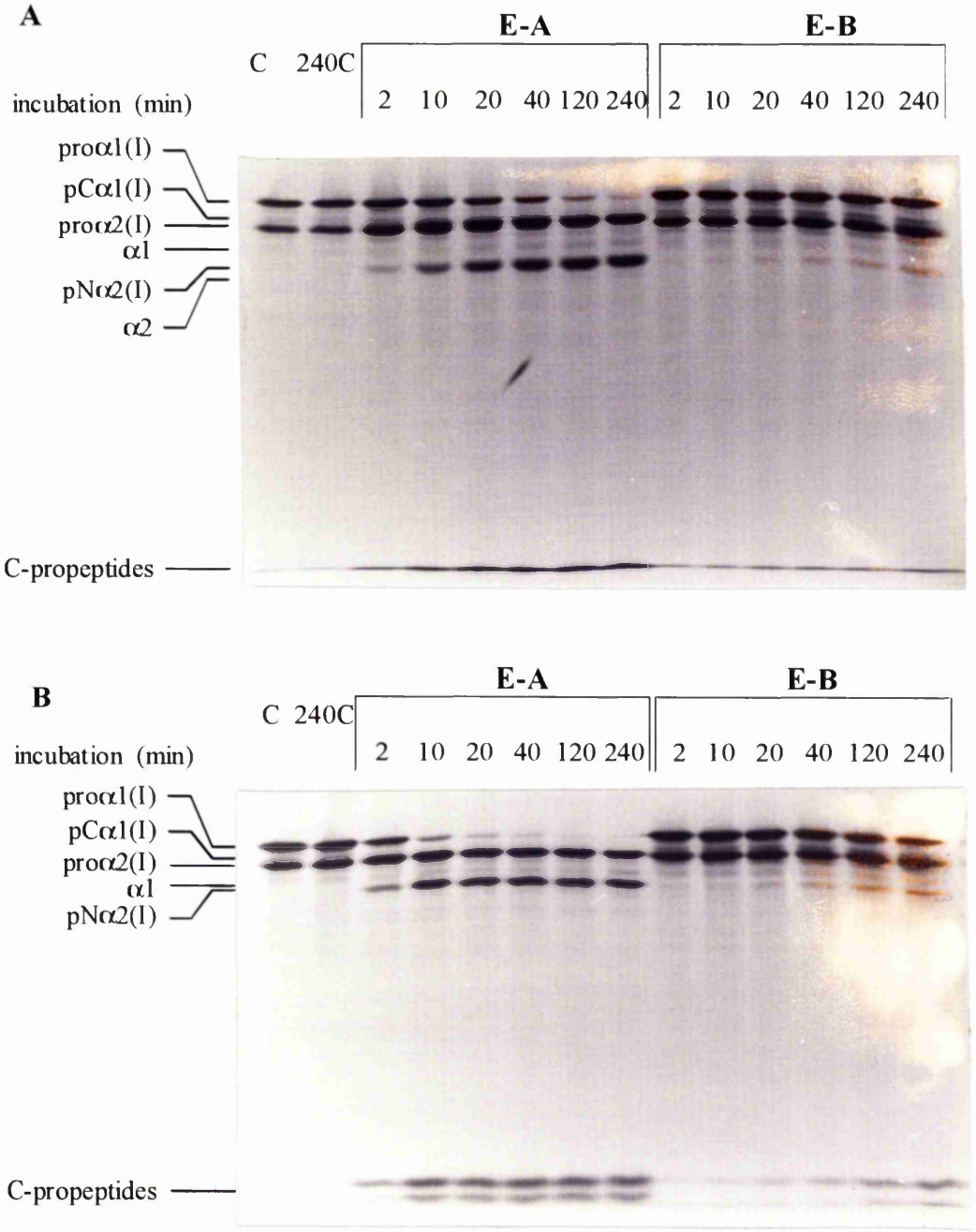
Samples (10 µl) from fractions (numbers as indicated) were added to 1 µg CET-procollagen (with 0.02% (w/v) DS), incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. ***Lane C***, 1 µg procollagen incubated without enzyme. ***A***, fractions from first protein peak (E-A peak). ***B***, fractions from eluted peak (E-B peak). The presence of pCα1(I) suggests N-proteinase activity in active B fractions.

ultrafiltration. The second pool (110 ml, 10.4 mg protein) named E-B, was concentrated and 72% of protein and activity recovered. The concentrated samples were assayed using both the enhanced and the basic electrophoretic assay (Figure 3.21). From the autoradiographs shown, E-A was more active compared to E-B and with the addition of DS cleavage of procollagen was enhanced five fold in the E-A sample. Units of enzyme activity in the two samples were determined by plotting the % cleavage of proα2(I), (calculated using equation 1), against time of incubation (Figure 3.22). The initial rate of cleavage was determined and from this units/ml calculated. Total units of activity in the E-A and E-B samples were 1128 and 34 respectively, which gave a total recovery of activity from the heparin-Sepharose column of 40.5%. The recovery of activity over the three affinity columns was 17%, and a 25.7 fold purification of E-A was achieved. Approximately 40 μg of C-proteinase was partially purified from a starting volume of 6.6 L. Concentrated E-A from this purification was allocated for characterisation and inhibitor studies, and E-B was passed over a Sephacryl-300 gel filtration column, as described below.

3.4.4.4 Sephacryl-300 gel filtration

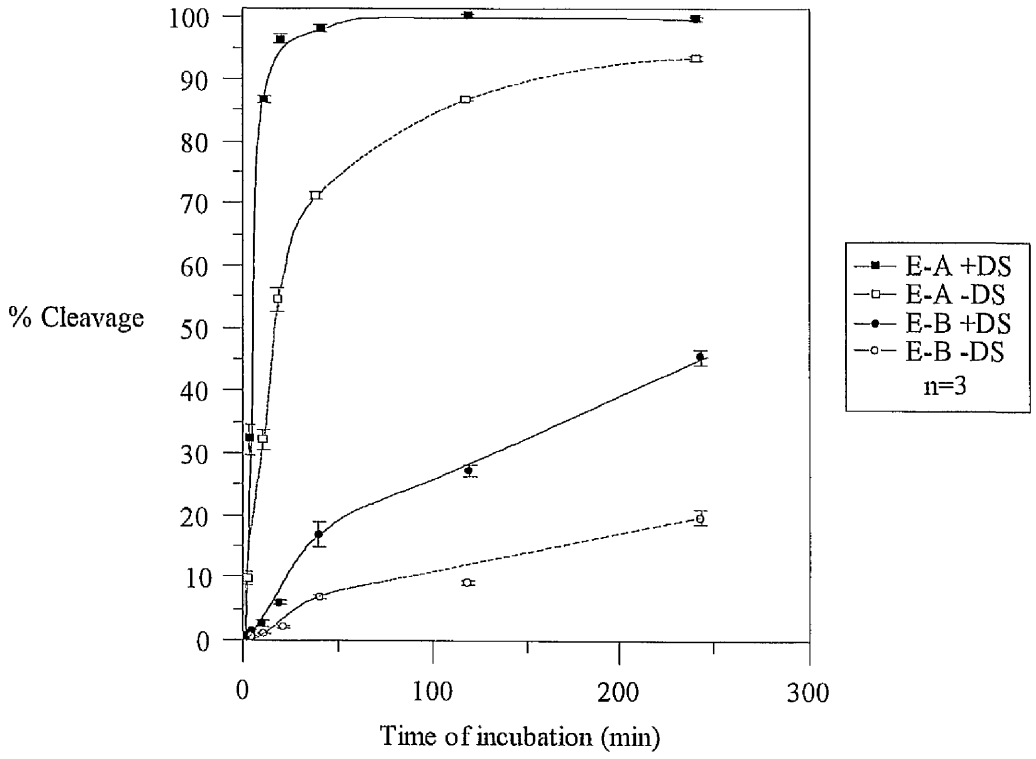
C-proteinase E-B was dialysed into S-300 equilibration buffer and loaded on to a S-300 column (Figure 3.23). Samples from fractions were assayed using the enhanced assay, and a small amount of activity (10 units total) was recovered. No protein was detected by silver staining after 100 μl samples of fractions were resolved on a 7% separating SDS-PAGE gel.

Figure 3.21 Assay Time Courses of Concentrated Human E-A and E-B C-proteinase



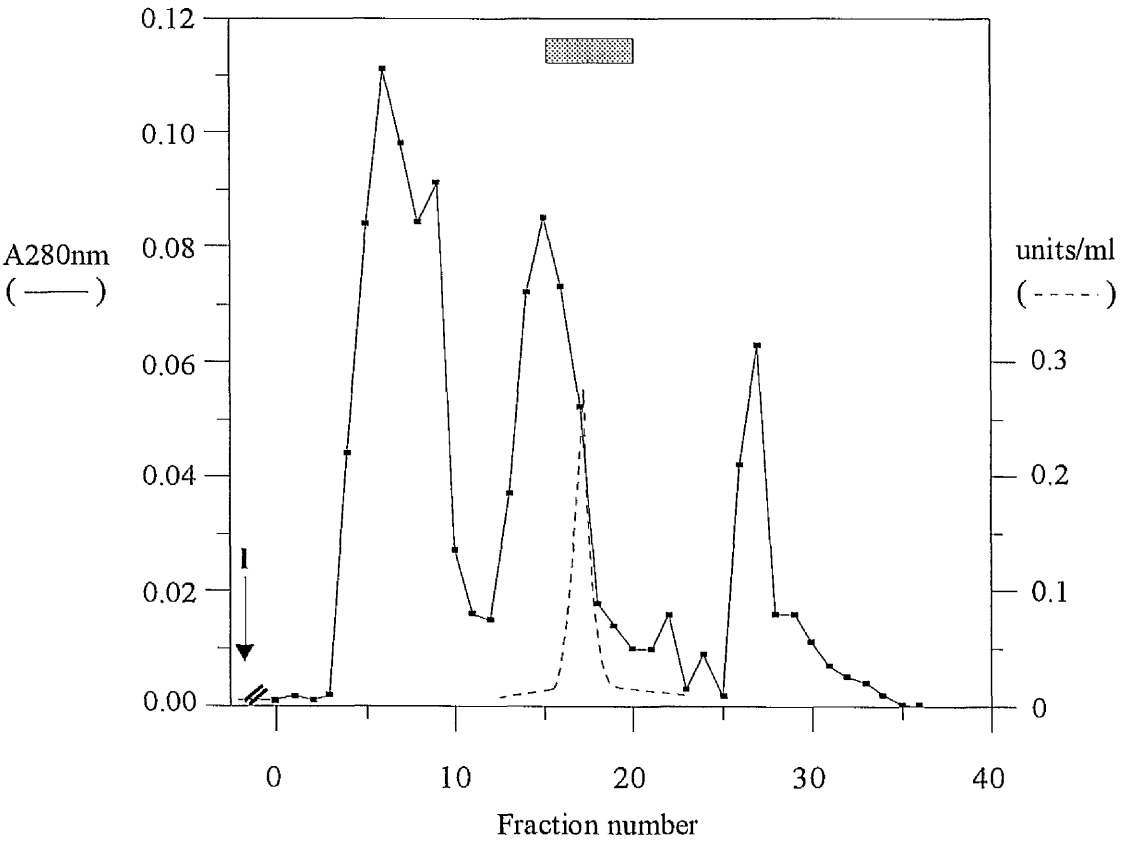
C-proteinase samples (10 µl) from concentrated E-A and E-B were incubated with 1 µg CET-procollagen, for 2, 10, 20, 40, 120 and 240 min at 37 °C, and analysed by SDS-PAGE (7% separating) under reducing conditions, and proteins detected by fluorography. ***A***, assay with no DS added. ***B***, assay with 0.02% w/v DS added to reaction mixtures. ***Lane C***, 1 µg CET-procollagen unincubated; ***lane 240C***, 1 µg CET-procollagen incubated for 240 min.

Figure 3.22 Determination of Units of Activity of Human C-proteinase (± DS)



Intensities of bands from the C-proteinase electrophoretic assay of concentrated human E-A and E-B, ± 0.02% (w/v) DS (Figure 3.21), were determined by densitometry. The % cleavage of proα2(I) was plotted against time of incubation, and initial rates (linear part of graph) of cleavage were to calculate units of activity; where 1 unit is the amount of enzyme which cleaves 1 μg of type I procollagen in 1 hr at 37 °C. E-A (-DS) 205±12 units/ml, E-A (+DS) 970±78 units/ml, E-B (-DS) 5±1.8 units/ml, E-B (+DS) 13.6±0.33 units/ml.

Figure 3.23 Sephacryl-300 Gel Filtration of Human C-proteinase (E-B)



Pooled E-B fractions from heparin-Sepharose chromatography were dialysed into S-300 equilibration buffer and applied to a S-300 gel filtration column (2.5cm x 110cm) at a flow rate of 15 ml/hr (**I**). The absorbance was determined for alternate 10 ml fractions. Activity was determined by electrophoretic assay. Samples (10 µl) from alternate fractions were added to 1 µg CET-procollagen, incubated for 4 hr at 37 °C, analysed by SDS-PAGE and proteins detected by flurography. Units were calculated (equation 1) and the units/ml plotted (dotted line). Fractions containing active C-proteinase (15-20, shaded box) were pooled.

3.4.4.5 Synopsis of Purification of Human C-proteinase

The successful purification of human C-proteinase, from 6.6 L of media from cultured MG63 cells, is summarised in Table 3.3. The units of activity were determined using the basic SDS-PAGE method, and % recovery and specific activity (units/mg), determined at each stage of purification. Active E-A, and to a lesser extent E-B were partially purified using the protocol of 3 affinity columns, devised for purifying chick C-proteinase. Levels of C-proteinase were slightly greater in crude MG63 cell growth media (1.6 unit/ml) compared to chick tendon organ culture media (1.3 unit/ml). However 84% of enzyme activity was recovered from the Green A column, compared to 83-90% in the chick purifications, and significant losses (~50% enzyme activity) were recorded over the concanavalin A-Sepharose column. With chick enzyme this second stage gave a 6-7.3 fold purification of C-proteinase, whereas with human enzyme purification, only 1 fold was achieved. Two forms of C-proteinase were separated by heparin-Sepharose chromatography, with 39% of activity eluted in the first active peak (E-A) and 2% in the second (E-B). Unlike in the purification of chick C-proteinase only the Green A DyeMatrex chromatography resulted in significant purification.

Table 3-3 Summary of Human C-proteinase Purification from MG63 cells

	Volume (ml)	Protein (mg)	Activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
Crude media	6600	1452	6800	100	4.68	1
Green A Concentrated pool	62.5	61	5720	84	93.8	19.6
Concanavalin A Concentrated pool	30	29.1	2880	50.3	98	20.4
Heparin-Sepharose Concentrated pool	5.5	9.2	1128	39.3	123	25.7

Activity was determined using the basic electrophoretic assay, where samples were analysed by SDS-PAGE and units calculated by plotting % cleavage of proα1(2) against time.
Recovery is % recovery of activity units after concentrating the pool, respective of activity obtained from previous column
Fold purification is determined by comparing specific activities (units/mg)

3.5 CHARACTERISATION OF HUMAN PROCOLLAGEN C-PROTEINASE

3.5.1 Effects of C-proteinase Concentration on Procollagen Cleavage

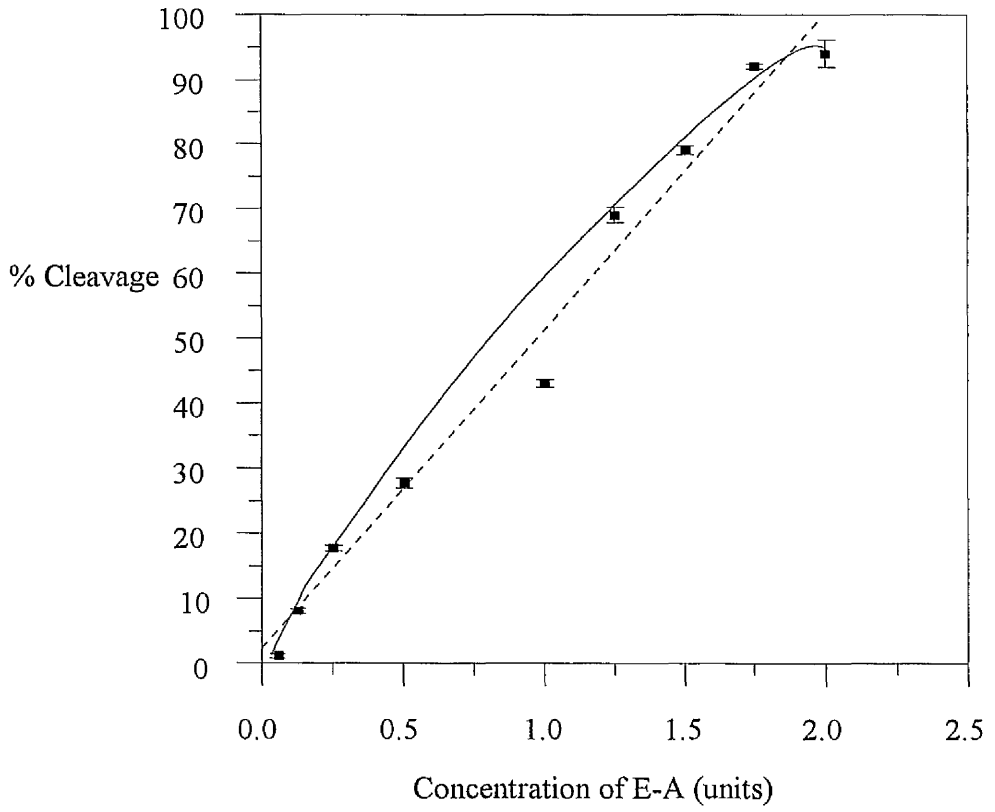
Prior to characterisation of the enzyme the linearity of the assay with increasing amounts of human enzyme was tested in a series of experiments using 0-2 units of human E-A (Figure 3.24). Activity in each sample was expressed as % cleavage, relative to that observed with no enzyme. Overall a linear cleavage of procollagen was observed with increasing concentrations of C-proteinase. Where the linear regression equation was determined as $y = 48.9x + 2.31$ and the correlation coefficient was calculated as 1.26. At the low levels of enzyme (<0.5 units) cleavage was directly proportional to concentration.

3.5.2 Sequence Analysis of Cleaved C-propeptides

Chick C-proteinase was shown to cleave the Ala-Asp bond in both proα1(I) and proα2(I) chains *in vitro* (Hojima *et al.*, 1985), confirming observations of cleavage *in vivo* (Dickson *et al.*, 1981). To characterise the human enzyme purified from MG63 culture medium, complete cleavage of procollagen using the enhanced assay conditions was carried out, and the C-propeptides subjected to N-terminal amino acid sequencing.

Experiments had shown that 95% cleavage of human procollagen was possible with the addition of 0.02% (w/v) DS (Figure 3.5). To achieve this percent cleavage human and chick procollagen (100-150 μg) were incubated with 0.02% (w/v) DS overnight in the presence of human E-A. The cleaved proteins were separated by SDS-PAGE and

Figure 3.24 Activity of Human E-A



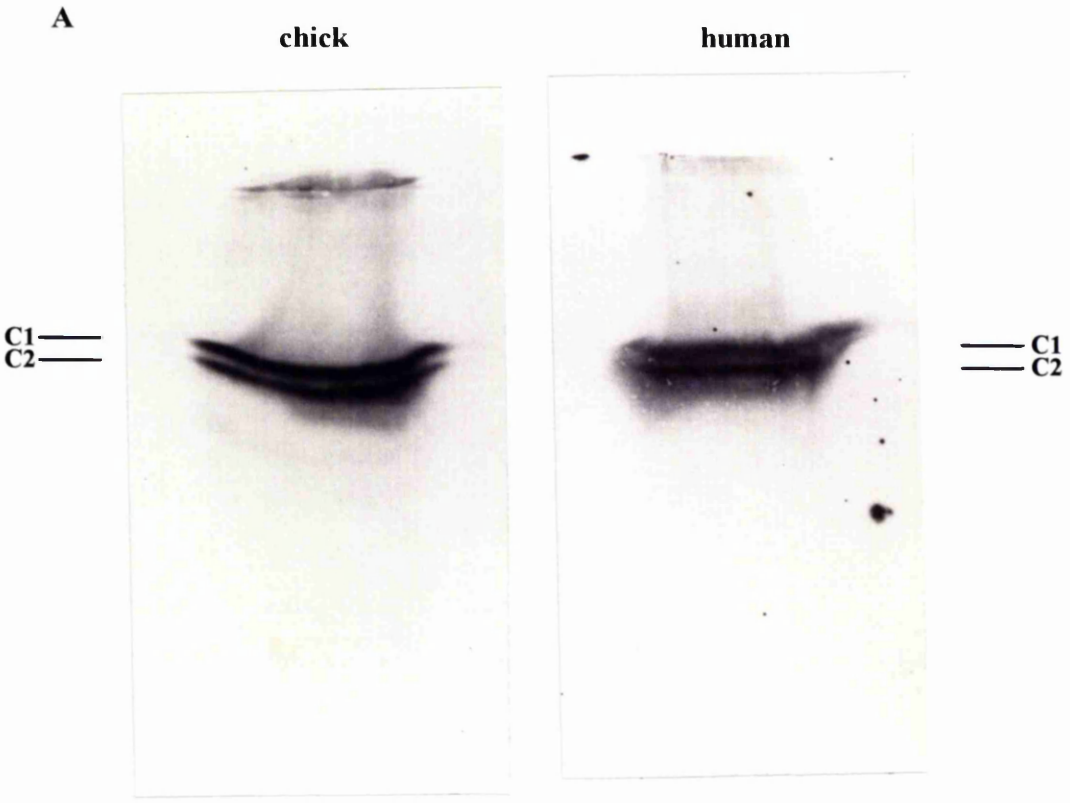
Samples of C-proteinase E-A (0-2 units), from heparin-Sepharose chromatography, were incubated with 1 μg CET-procollagen for 4 hr at 37 °C, and analysed by SDS-PAGE (7% separating), under reducing conditions and separated proteins detected by fluorography. Activity was expressed as % cleavage relative to procollagen incubated without enzyme, under identical conditions. The group number was 2. Dotted line indicates linear increase of % cleavage with enzyme concentration, and the solid line indicates the actual line of cleavage.

blotted to PVDF using a 10 mm CAPS, pH 11, and 10% (v/v) methanol transfer buffer, and stained with Coomassie blue. Autoradiographs of the blots were obtained and the C1 and C2 -propeptides identified (Figure 3.25, A). Coomassie blue staining of gels after blotting revealed that all protein had been transferred. The N-terminal C1 propeptide sequences in the chick and human were identical (figure 3.25, B). The cleaved bond, -Ala-Asp-, is identical to that cleaved by the chick enzyme *in vitro*. The first three amino acids in the C2 propeptide human sequence are identical to the chick, and to the sequence of cleaved chick procollagen with chick enzyme, again indicating that the enzyme purified from MG63 culture medium cleaved chick and human procollagen at the expected -Ala-Asp- site (Dickson *et al.*, 1981).

3.5.3 Class Specific Inhibitors

Inhibitors from the classes of proteinases (metallo-, serine and cysteine) were incubated at a range of concentrations with human E-A, from the heparin-Sepharose column, and samples analysed by SDS-PAGE. Inhibition of % cleavage was relative to controls with no inhibitor present. The metal chelators, EDTA and *o*-phenanthroline inhibited human C-proteinase (Table 3.4). *O*-phenanthroline was the most potent, inhibiting C-proteinase 100% at 3 mM. These results confirm that C-proteinase is a metalloproteinase, presumably dependent on a zinc ion cofactor at the catalytic domain for activity. Specific serine inhibitors had minimal effect, with PMSF inhibiting activity 11% at 5 mM, and Soybean Trypsin inhibitor having no effect (Table 3.4). The cysteine inhibitor N-ethylmaleimide, which reacts with sulfhydryl groups, was also ineffective at 5 mM and causing 6% inhibition at 10 mM (Table 3.4).

Figure 3.25 Sequence Analysis of C-Propeptides Cleaved from Type I Procollagen by Human C-proteinase



B

(C1) Hu	**---Asp-Asp-Ala-Asn-Val-Met-Arg-Asp-Arg-Asp---**
Ch	**---Asp-Asp-Ala-Asn-Val-Met-Arg-Asp-Arg-Asp---**
(C2) Hu	**---Asp-Gln-Pro-Asn-Val-Met-Arg-XX-Asp-Tyr---**
Ch	**---Asp-Gln-Pro-Ser-Leu-Arg-Pro-Lys- Arg-Tyr---**

Chick (150 μg) and ***human*** procollagen (110 μg) were cleaved with 6.6 units of purified human C-proteinase (E-A), in the presence of 0.02% (w/v) DS, at 37 °C, overnight. ***A***, The C1 and C2 -propeptides from complete procollagen cleavage were analysed by SDS-PAGE (15% separating), under reducing conditions, and separated C-propetides detected by autoradiography. ***B***, ***C1*** and ***C2*** -propeptides were excised from PVDF membrane: human (Hu) and chick (Ch) and amino acid sequence data from N-terminal sequencing is shown. The arrow indicates the point of human C-proteinase cleavage in the proα1(I) and proα2(I) chains.

Table 3-4 Class Specific Inhibitors on Human Procollagen C-proteinase Activity

Inhibitor	Concentration	Inhibition (%)
EDTA	5 mM	72
	10 mM	100
	20 mM	100
o-Phenanthroline	1 mM	82
	3 mM	100
PMSF	5 mM	11
	10 mM	4
Soybean trypsin inhibitor	50 μg/ml	0
	100 μg/ml	0
N-Ethylmaleimide	5 mM	0
	10 mM	6

Specific class inhibitors incubated with 0.5 units of human E-A for 40 min
Inhibition expressed as a % inhibition of cleavage, determined by SDS-PAGE (7% separating) under reducing conditions, fluorography and densitometry of bands
Data from one experiment is shown

Table 3-5 Inhibition of Human C-proteinase by General Buffer Components

Inhibitor	Concentration	Inhibition (%)
α-methyl-D-mannoside	0.5 M	0
	0.95 M	18
Tris-HCl	0.1 M	69
	0.5 M	78
	1 M	95
NaCl	0.2 M	64
	0.5 M	77
	1 M	94

Data from one experiment is shown

3.5.4 Inhibition by Buffer Components

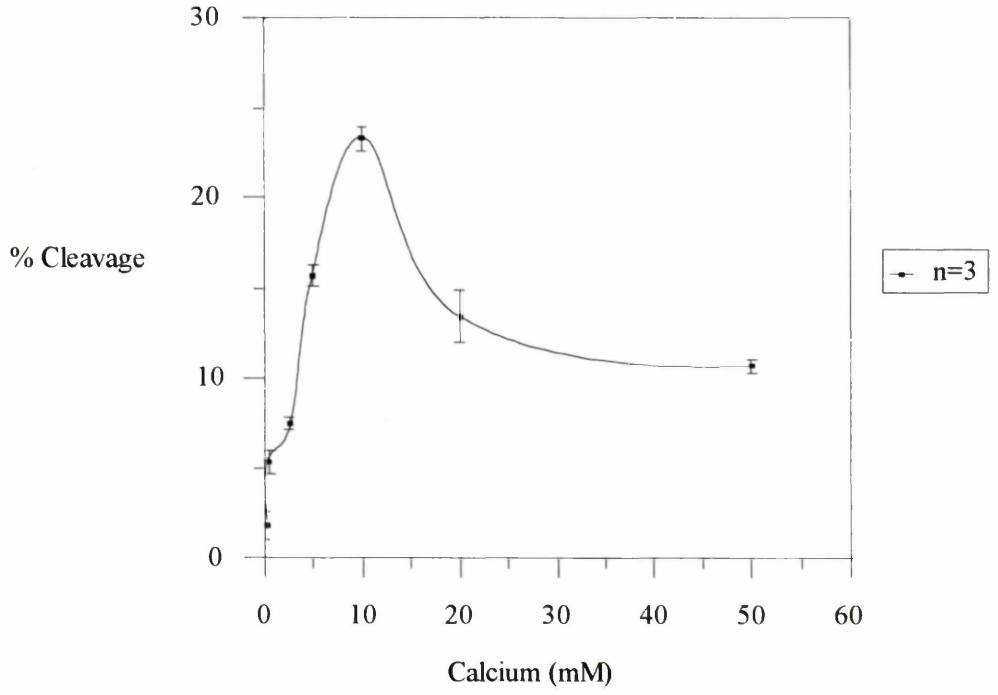
The electrophoretic assay was carried out in the prescence of human E-A with a range of concentrations of Tris-HCl, NaCl and α-methyl-mannoside, whilst maintaining the other assay buffer concentrations of 0.05 M Tris-HCl buffer, pH 7.5, 0.15 M NaCl, 5 mM $CaCl_2$ (Table 3.5). Samples were analysed by SDS-PAGE and inhibition of cleavage was respective of that cleaved with the usual buffer concentrations. Methyl-D-mannoside, the carbohydrate used to elute proteins tightly adsorbed to the concanavalin A-Sepharose column, inhibited activity 18% at 0.95 M and had no effect at 0.5 M. Tris-HCl concentrations of 0.1, 0.5 and 1 M inhibited activity with a dose dependent response with 1 M inhibiting activity 95%. A similar trend was seen with NaCl concentrations, as activity was inhibited from 64% (0.2 M) to 94% (1 M).

The results clearly demonstrate the necessity of diluting enzyme samples in the electrophoretic assay to concentrations of 0.05 M Tris-HCl and 0.15 M NaCl. Proteins are often eluted from affinity chromatography columns with >1 M NaCl and between 0.3-1 M Tris-HCl, and as long term effects of these chemicals on activity is unknown, it was important to purify the enzyme as quickly as time would allow.

3.5.5 Calcium Dependency

Human E-B, prior to pressure ultrafiltration, from the heparin-Sepharose column was used in this investigation as the elution buffer contained no $CaCl_2$. The enhanced assay was used on the sample (10 µl) taken from the 105 ml pool, and samples were analysed by SDS-PAGE, and the % cleavage plotted (Figure 3.26). There is a

Figure 3.26 Calcium Dependence of Human C-proteinase Activity



Human E-B, eluted from the heparin-Sepharose was added to 1 μg CET-procollagen, 0.02 % (w/v) DS and 0-50 mM $CaCl_2$, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. Intensities of the bands were determined by densitometry, and the % cleavage calculated (equation 1) and plotted.

significant 3 fold increase cleavage with 0.5 mM $CaCl_2$, with optimum activity requiring 5-15 mM. With 50 mM $CaCl_2$, activity was 1.5 fold greater than at 2.5 mM. The result is consistent with the calcium dependency of the chick enzyme (5-10 mM), illustrating the requirement of the calcium ion for activity.

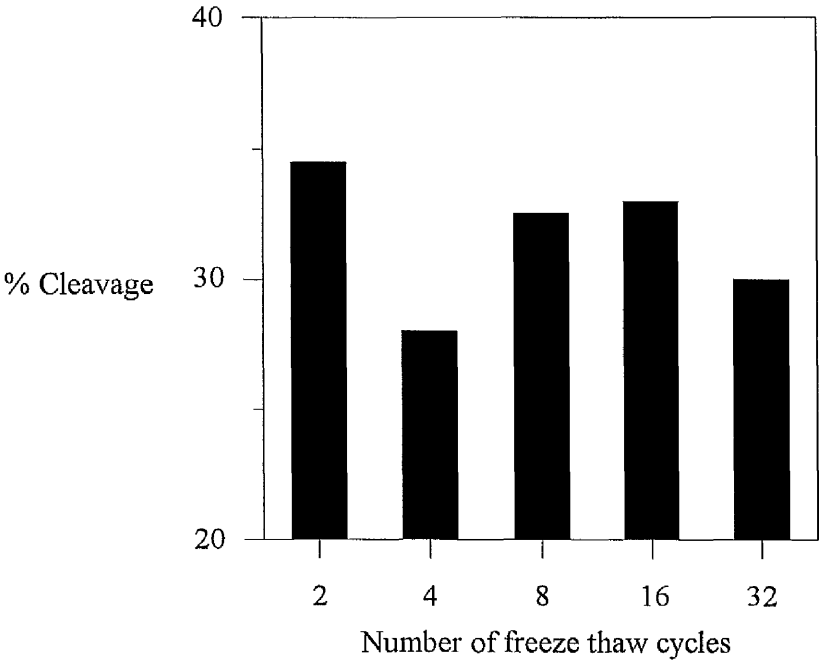
3.5.6 Freeze Thawing

Enzyme activity may have decreased in accordance with the number of times the stock sample was freeze thawed. Human E-A freeze thawed for up to 32 cycles, was assayed and units of activity determined by plotting the % cleavage (Figure 3.27). There was no significant effect on activity with freeze thawing, as after 2 cycles C-proteinase had cleaved procollagen by 34.5%, and after 32 cycles it was cleaved by 30%. As the stock would only be thawed out a maximum of 20 times losses due to freeze thawing were not considered important.

3.5.7 Long Term Storage Effects on Chick C-proteinase

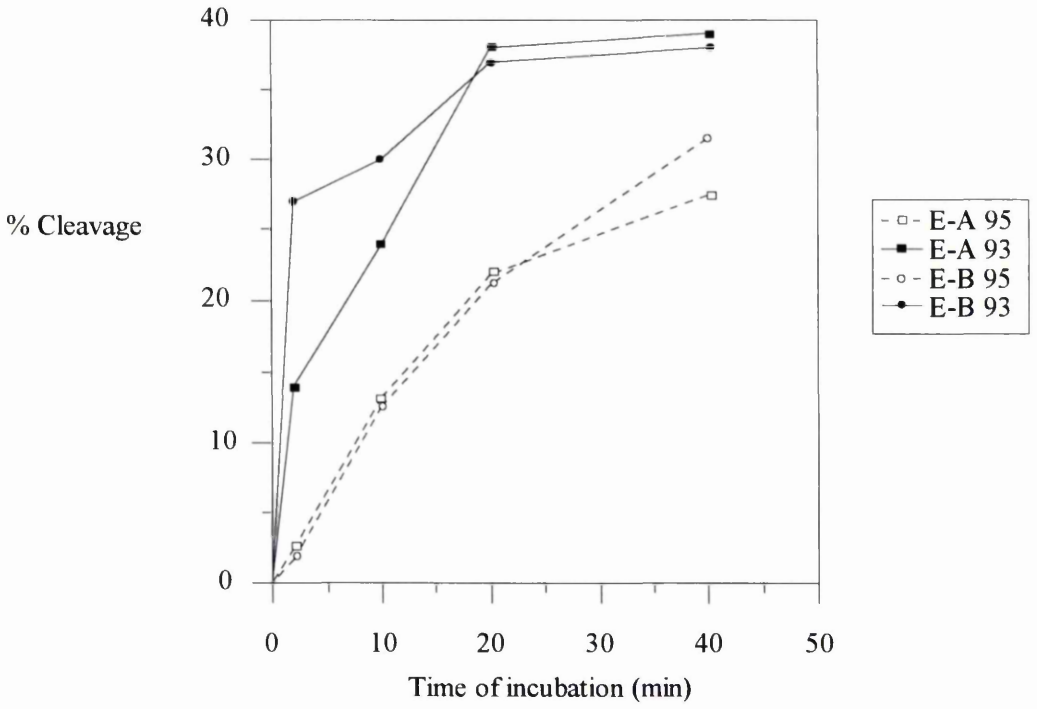
Storage of enzyme in 1 M NaCl and 0.3 M Tris-HCl buffer, pH 7.5, might be expected to effect activity. Activity of chick C-proteinase after two years of storage was compared to that at the time at purification. Chick E-A and E-B from heparin-Sepharose chromatography when purified in 1993 was assayed to determine units of activity. The same assay was repeated two years later under identical conditions. Samples (5 μl) were assayed using the basic electrophoretic assay and units determined by plotting the % cleavage and finding the initial rates of cleavage (Figure 3.28).

Figure 3.27 Freeze Thawing Sensitivity of Human C-proteinase



Human E-A (0.5 units), freeze thawed repeatedly for a number of cycles (shown in the box), was incubated with 1 μg CET-procollagen for 10 min at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. Intensities of the bands were determined by densitometry, and the % cleavage calculated (equation 1) and plotted. Data from one experiment is shown.

Figure 3.28 Storage Sensitivity of Human C-proteinase



Chick C-proteinase (5 µl, ***E-A*** and ***E-B*** before ***(93)***, and after 2 years storage***(95)***, at -20 °C) was incubated with 1 µg CET-procollagen for 2, 10, 20, and 40 min at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, and proteins detected by fluorography. Intensities of the bands were determined by densitometry and the % cleavage plotted. Data from one experiment is shown.

E-A and E-B, from initial rates of cleavage of 0.72 μg procollagen/hr, had activities of 145 units/ml. Compared to the activities of E-A and E-B in 1993 (625 and 720 units/ml) a loss of 80% activity was recorded. The same effect may occur with the human enzyme, although this was not investigated.

3.6 DISCUSSION

As discussed in earlier sections, the next major advances in understanding the characterisation of C-proteinase would result from comparison of primary sequence with known proteins and from studies with specific antibodies. Type I procollagen C-proteinase has been partially purified by a number of groups, and was first characterised as a 97-110 kDa single polypeptide protein, from chick embryo tendon, by Hojima *et al.*, (Hojima *et al.*, 1985). This was in contrast to a 80 kDa C-proteinase, purified from mouse fibroblasts (Kessler & Adar, 1989), which required a 55 kDa enhancer molecule for activity. As the chick C-proteinase did not require this enhancer for activity, and that chick embryo tendon had the highest and most reproducible levels of C-proteinase activity, it was decided to use this source initially in my studies. As a purification scheme had been established by Hojima *et al*, to purify C-proteinase from chick tendon (Hojima *et al.*, 1985) the first part of my thesis was to repeat this procedure and attempt to obtain sufficient purified chick C-proteinase to obtain a partial N-terminal amino acid sequence of the enzyme. This would be useful in preparing a foundation for the subsequent purification of the human enzyme.

Prior to the start of this work, a 'rapid assay' was used (Kessler & Goldberg, 1978) to determine the location of active C-proteinase in fractions eluted from the

chromatography columns. However, problems soon arose, with high background readings, probably due to non-specific cleavage of procollagen molecules. Even though it was quick to use, this unpredictability and inaccuracy warranted the use of the slower, but more specific SDS-PAGE assay. This electrophoretic assay was used throughout these studies, where specific cleavage of substrate was visualised on autoradiograms after treatment by flurography. Specific cleavage products, pNα1(I), and pNα2(I), were observed with active C-proteinase. However, during the earlier stages of purification and in small volumes, such as in cell culture studies, often levels of C-proteinase were too low to be detected. The addition of the neutral polymer 0.02% (w/v) DS, to the reaction sample, enhanced cleavage 2-5 fold by aggregating the procollagen, a phenomenon observed by Hojima *et al.*, (Hojima *et al.*, 1994). This was developed here and used as a more sensitive way of detecting C-proteinase.

From initial studies it was concluded that 300 doz chick embryos would be required to obtain enough C-proteinase to obtain N-terminal sequence. As the availability of fertilised eggs was limited to a delivery of once a week, it would take 3 months to generate enough starting material. The first two purifications yielded only 20-50 units of active C-proteinase. It was discovered that adding solid $CaCl_2$ to the starting media results in the loss of C-proteinase activity, probably due to the co-precipitation with insoluble calcium salts with DMEM media components, such as phosphate.

To increase the rate of generation of starting media, dissection was increased to 50 doz embryos per week, however, a number of unexpected complications resulted in 50% cultures being contaminated, and low enzyme yields. The low yields were probably

due to proteases released by contaminating bacteria present in day one cultures, but which were not apparent until days two or three. A new, improved, method of culturing the tendons was soon developed, where the tendon yield was split into half and cultured in separate Erlenmeyer flasks containing 500 ml of DMEM. Contamination was reduced to <10%, and after a significant amount of time and effort, partially purified chick C-proteinase was prepared.

The Green A DyeMatrex column purified C-proteinase 15 fold, the most significant step, separating the enzyme from 90-95% of loaded protein. Major losses of activity were always observed with the second column, the concanavalin A-Sepharose, where up to 50% of protein was lost during pressure ultrafiltration. Even using a greater concentration of 1 M α-D-methylmannoside, and increasing the length of stirring did not increase recovery. Two active forms of C-proteinase were always separated by heparin-Sepharose chromatography. In purification number four, the ratio of E-A:E-B was 10:1, whereas in the fifth purification the ratio was ~1:1. Differences in the ratio may be due to an artefact of one of the purifications as only two purifications were completed. These differences in ratio may suggest that E-B is a degradative product of E-A or one is a precursor of the other. As E-B is only eluted with a higher NaCl concentration (0.2 M instead of 0.05 M), it may have a different level of glycosylation and a higher affinity for heparin. In total 400μg of 4-6% pure C-proteinase was purified from a starting volume of 6.4 L. Therefore the initial concentration of C-proteinase in tendon was 0.4μg/g of tendon. Unlike in the work carried out by Hojima *et al.*, after gel filtration using Sephacryl S-300, the resulting enzyme, E-A C-proteinase, was not homogenous. Instead, the major protein observed was a 62 kDa

protein. This, in hindsight, could possibly have been the enhancer molecule, which was characterised by Takahara *et al.*, (Takahara *et al.*, 1994a).

One way of proceeding with the project would have been to adopt a molecular biology approach. Where an oligonucleotide sequence from C-proteinase would be used to design primers for reverse transcription-PCR, to screen cDNA libraries. This would have yielded the primary sequence for comparison with known proteins. An N-terminal sequence of six residues was obtained by concentrating 0.5 mg of protein, in the chick E-A sample, by freeze drying, separating by a 7% (w/v) polyacrylamide separating SDS-PAGE and blotting to PVDF. The sequence, however, was blocked at the N-terminal end. This may have been due to a low yield on the first cycle possibly because a significant proportion of the N-terminal had cyclised and therefore was resistant to the sequencing chemistry. As the sequence was short and had significant codon degeneracy it would not have been specific enough to isolate C-proteinase cDNAs.

The five attempts at purifying chick C-proteinase to homogeneity, utilised the dissection of 1500 doz chick embryos resulted in only a 4-6% pure C-proteinase. This approach was abandoned in favour of finding a better source of enzyme, preferably from cells in culture, which would reduce the need for dissection of chick embryos, and would speed up progress.

Various human cell lines were investigated because of their ability to produce mature collagen. The MG63 and SAOS-2 osteosarcoma cells had the greatest levels of C-

proteinase, however, the SAOS-2 cell line, also had high levels of N-proteinase in the growth media, and so, the MG63 cell line was used as the source of human enzyme. The cells were shown to maintain secretion of C-proteinase into the media at a stable concentration for up to 4 weeks. As the cell line is transformed, bulk culturing of the cells was straightforward with cells requiring passaging every 3 days with a split ratio of 1:3.

Prior to scaling up cell culturing, a number of pilot purification attempts were carried out, showing that the same columns, could be used to obtain human enzyme. C-proteinase, levels in MG63 cell growth media were slightly greater (1.6 unit/ml) compared to those found in the chick organ culture media (1.3 unit/ml). Even though this was not a major advancement, the relative ease of cell culture compared to tendon isolation and culturing, meant that 40 flasks could be maintained, each with 25 ml of media, and harvesting would take place every four days. Therefore, generating 6L of media would take half the time, compared to that in the chick purifications.

Human C-proteinase was partially purified, for the first time, from media from MG63 cells (6.6 L) containing 6800 units of activity, and 1452 mg of protein which was collected over one month (Table 3.3, shows a summary of purification scheme). Green A DyeMatrex chromatography purified and concentrated C-proteinase 20 fold, however, 16% of activity was lost, similar to the 10-17% loss seen with chick purifications. This may have been due to column overloading and, or the presence of FCS, which may have inhibited a small percentage of C-proteinase. Concanavalin A-Sepharose chromatography only purified the C-proteinase one fold, with a 50% major

loss of activity that was loaded on to the column. This was the most critical step, inferring the largest loss of activity during purification, and even with the addition of 1 M α-D-methylmannoside in the elution buffer only a further 5% of protein was eluted from the column. Usually 20% of protein was not recovered from the column. This could be explained by a high glycosylation level, with the carbohydrates having high affinity for the concanavalin A-Sepharose.

Heparin-Sepharose also separated C-proteinase into two forms, E-A and E-B. However, the purification factor would have been higher if the 60% loss of activity had been minimised. Possibly the bound form of human C-proteinase, E-B, had a higher affinity for heparin due to greater levels of glycosylation or other interactive domains, different to those found on the chick enzyme. The ratio of elution of E-A:E-B was 33:1, very different to the 1:1 chick ratio. The E-A human form may be the dominant variant, with the E-B, having a minor role in the processing of procollagen. If E-B was a degradative form of E-A, the results would indicate that little enzymatic processing was occurring in the cell system. Unfortunately, even though the initial experiments were promising, only 1130 units of C-proteinase were purified, giving a specific activity of 123 units/mg. This was a 10 fold reduction in yield compared to that observed in the chick C-proteinase purification. With only ~40μg of C-proteinase being purified, from each purification, it was difficult to produce sufficient enzyme for characterisation experiments. The E-A sample was further purified by S-300 gel filtration, however, all activity was lost and no proteins were detected by silver stain, after electrophoresis of column fractions.

Partially purified E-A human C-proteinase from the heparin-Sepharose chromatography, totally cleaved both human and chick procollagen, with 0.02 % (w/v) DS, which allowed N-terminal sequencing of cleaved C-propeptides. In both cases the sequences of the excised C-propeptides were identical to that seen with chick C-proteinase cleavage, confirming that the activity in the purified protein was procollagen C-proteinase, and that the human form also cleaves at the ^{123}Ala-^{124}Asp bond of human and the Ala-Asp bond of chick procollagen.

The human form was also a neutral metalloproteinase. As the metal chelators *o*-phenanthroline and EDTA inhibited activity 100% at 3 and 10 mM respectively. Also cysteine and serine protease inhibitors, had no effect on activity. Tris-HCl and NaCl inhibited activity by 64-69% at 0.1 M and 0.2 M concentrations. The long term effects of C-proteinase being eluted in high concentrations of these buffer components is unknown and may have been contributory to the loss of activity over the four columns. Human C-proteinase, like the chick C-proteinase, requires 5-15 mM Ca^{2+} for optimal activity.

4. RESULTS, PART II: EXAMINATION OF THE EFFECTS OF CYTOKINES AND GROWTH FACTORS ON C-PROTEINASE ACTIVITY IN HUMAN CELLS IN CULTURE

4.1 INTRODUCTION

Procollagen C-proteinase was successfully purified from MG63 culture media on a mass scale, using 6.6 L of medium. However only 1162 units of enzyme activity (~40 μg of C-proteinase) was recovered from starting material containing 6800 units. Understanding the extracellular factors which regulate the expression of C-proteinase in MG63 cells, could allow optimisation of the culture conditions used in the generation of this enzyme. If the rate of C-proteinase synthesis could be upregulated in MG63 cells the purification yields of active C-proteinase would also increase. To investigate effects on C-proteinase synthesis, a cell system was established and C-proteinase activity was determined using the SDS-PAGE assay methods. Effects of seeding density, volume, media, and ascorbate were all investigated before the cytokine studies progressed. Cytokines for this study were chosen because of their known effects on collagen synthesis in other cell systems, and with a working hypothesis that they may also be expected to regulate C-proteinase synthesis in a co-ordinate manner.

4.2 OPTIMISING GROWTH CONDITIONS

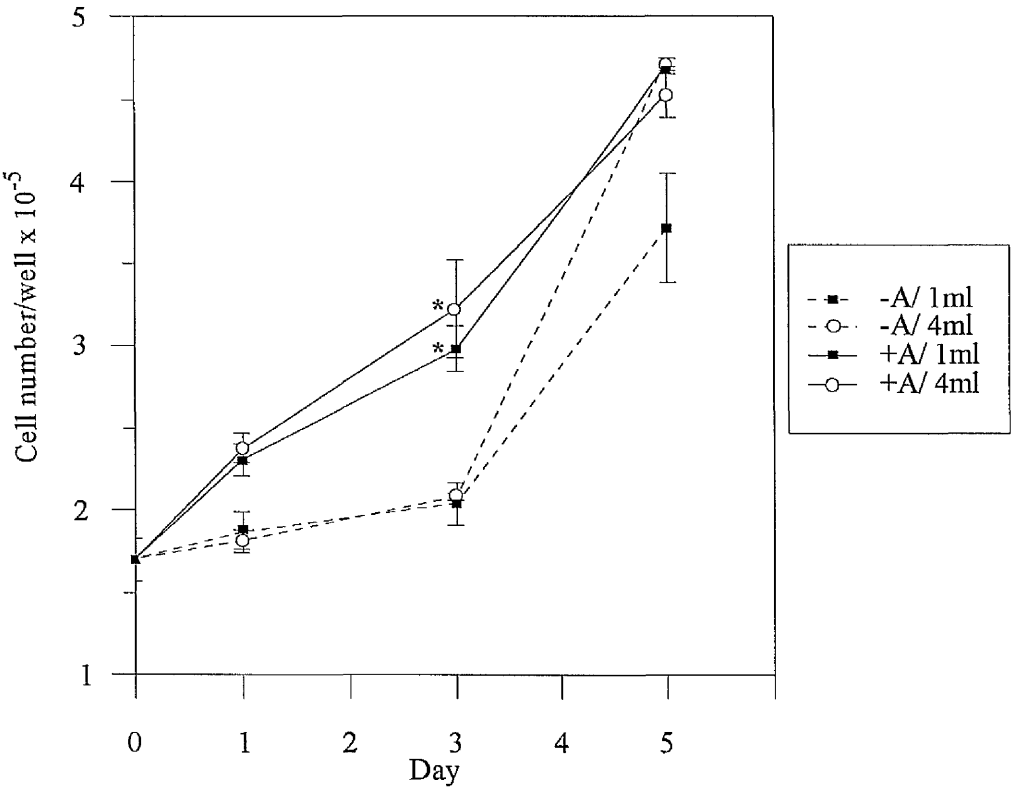
MG63 cells were seeded, in Costar 6 well plates, at a range of dilutions in 4 ml of DMEM culture medium and the number of adherent cells counted every day using a haemocytometer. Cells seeded at $<0.6 \times 10^5$ were slow to populate the plates but cells seeded at $>2 \times 10^5$ rapidly reached confluency within 24 hr. From these observations, experiments were set up with cells seeded at either 0.7 or 1.5×10^5.

Cells were seeded at 1.5 x10^5 /well in 6 well plates in 1 ml DMEM medium, containing 10% (v/v) FCS. After 24 hr, media was removed, cells were washed, and 1 or 4 ml of FCS free DMEM medium containing Nutridoma (a serum free supplement) with and without added ascorbate. The cells were counted at days 1, 3 and 5 after adding the test medium (Figure 4.1). The volume of media had no significant difference on cell number, whereas the presence of 50 μg/ml ascorbate stimulated cell growth. After 72 hr, cells in the presence of ascorbate had multiplied to 2.9 and 3.1 x 10^5 with 1 and 4 ml of media respectively, both significantly different compared to the control. No significant difference was seen at day 5. Ascorbate was used in media throughout the cell study, to optimise cell growth.

In studies to determine the effects of seeding density and volume and type of media on C-proteinase synthesis media was assayed using the enhanced electrophoretic assay (Figure 4.2) in triplicate. Statistical significance was calculated using the Student's t test.

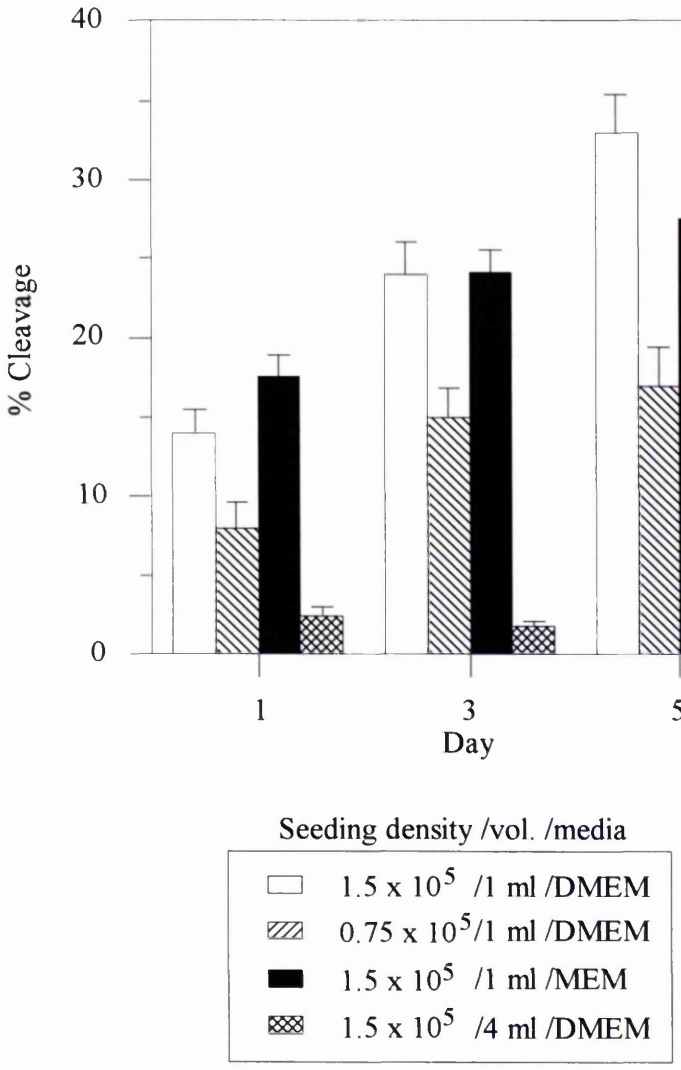
No significant difference in C-proteinase activity was observed between cells cultured in DMEM and MEM complete media. Activity was enhanced by 26% at day 1 with the MEM medium, however by day 5, cells grown in DMEM complete medium had secreted 19% more active C-proteinase than the cells grown in MEM. It was decided to study cells grown with the DMEM medium, to maximise nutrient levels available for metabolising cells. C-proteinase levels were higher in samples of media, from cells grown in a final volume of 1 ml, compared to 4 ml, at all time points (Figure 4.2).

Figure 4.1 Effects of Ascorbate and Volume of Media in Well on MG63 Cell Proliferation



MG63 cells where cultured in 1 or 4 ml of FCS free DMEM, supplemented with Nutridoma, with or without 50 μg/ml ascorbate (±**A**) for 1, 3 and 5 days. The viable cell number was determined using a heamocytometer and trypan blue, and the group number per test point was 3. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).

Figure 4.2 Effects of Seeding Density, Volume and Type of Media on MG63 Cell C-proteinase Production



MG63 cells, where cultured for 24 hr in FCS-supplemented DMEM media, washed and then grown under varying conditions in Nutridoma supplemented complete DMEM media supplemented with 50 μg/ml ascorbate, for 1, 3 and 5 days. Samples of media (89 μl) where assayed for C-proteinase using the enhanced electrophoretic assay for 4 hr, and samples subjected to SDS-PAGE (7% separating) under reducing conditions, and fluorography. Intensities of proα1(I) and pNα2(I) proteins determined by densitometry, where used to calculate % cleavage. ***Legend*** indicates seeding density of cell/well, volume of media added after seeding, and the type of media used. The group number per test point was 3. Error bars indicate SEM.

The levels of C-proteinase observed were significantly lower with 4 ml of DMEM media (1.8 and 2.4% cleavage), compared to 1 ml, (14 and 24.1% cleavage), at days 1 and 3 respectively. Hence to ensure C-proteinase was detectable from day 1, in growth factor studies on MG63 cell growth and enzyme production, the final volume of test medium was 1 ml. The rate of synthesising C-proteinase is significantly enhanced at all three time points when cells are seeded at the higher concentration of 1.5×10^5. This effect is a result of the increase in cell cycling and cell numbers (Figure 4.1), with a linear increase in C-proteinase ensuing. At day 5, cells seeded at 0.75×10^5, were synthesising stable levels of enzyme, whereas cells with the higher seeding density were obviously still rapidly secreting active C-proteinase into the media.

From these two experiments, effects of growth factors on MG63 cell growth, C-proteinase synthesis and activity, and collagen deposition, were studied on cells seeded at 1.5×10^5, in 6 well Costar plates, in a final volume of 1 ml of DMEM medium, containing 50 μg/ml ascorbate and 1%(v/v) Nutridoma.

4.3 GROWTH FACTOR STUDIES

A selection of growth factors (IL-1β, IL-4, EGF, FGFb and TGFβ1) were chosen which have been shown in fibroblasts to affect proliferation, collagen synthesis and in turn extracellular enzyme synthesis involved in processing procollagen. A cell system was designed from the previous experiments in section 4.2 and is described below. After 24 hr, adherent MG63 cells were washed twice with Nutridoma supplemented DMEM complete medium and left for 4 hr. One ml of medium containing three

concentrations of growth factor was then added in triplicate wells. For each growth factor examined, a total of 108 wells were seeded (12 for each parameter at each time point). Cell growth was determined using the MTT assay, collagen deposition in the cell layer determined by using the SIRCOL collagen assay, and C-proteinase activity determined by assaying 89 μl samples from the media using the enhanced electrophoretic method. Significant differences were calculated using the Student's t test, relative to untreated control cells.

4.3.1 Effects of Interleukin-1β

IL-1β is a monokine expressed by transformed B and T cell lines, and by osteoblasts, monocytes and fibroblasts in response to a variety of stimulants (Oppenheim, *et al.*, 1986). Its main biological functions include matrix degradation, by increasing MMPs, plasminogen activator and prostaglandin E_2 production in chronic inflammation (Mizel *et al.*, 1981), and is a potent stimulator of cartilage and bone resorption in a number of diseases including rheumatoid arthritis and periodontal lesions (Oshima *et al.*, 1994). However, in human cultured fibroblasts from scleroderma lesions, IL-1β stimulates the proliferation of cells and activates the pretranslational expression of types I and III collagen genes (Kähari *et al.*, 1987). The effects of IL-1β on MG63 cells had previously not been investigated so a wide range of concentrations (0.5, 10 and 20 ng/ml) were used in this cell system.

The initial rates of proliferation with IL-1β were similar, but by day 3 all treated cells were undergoing mitosis at significantly slower rates compared to the control (Figure

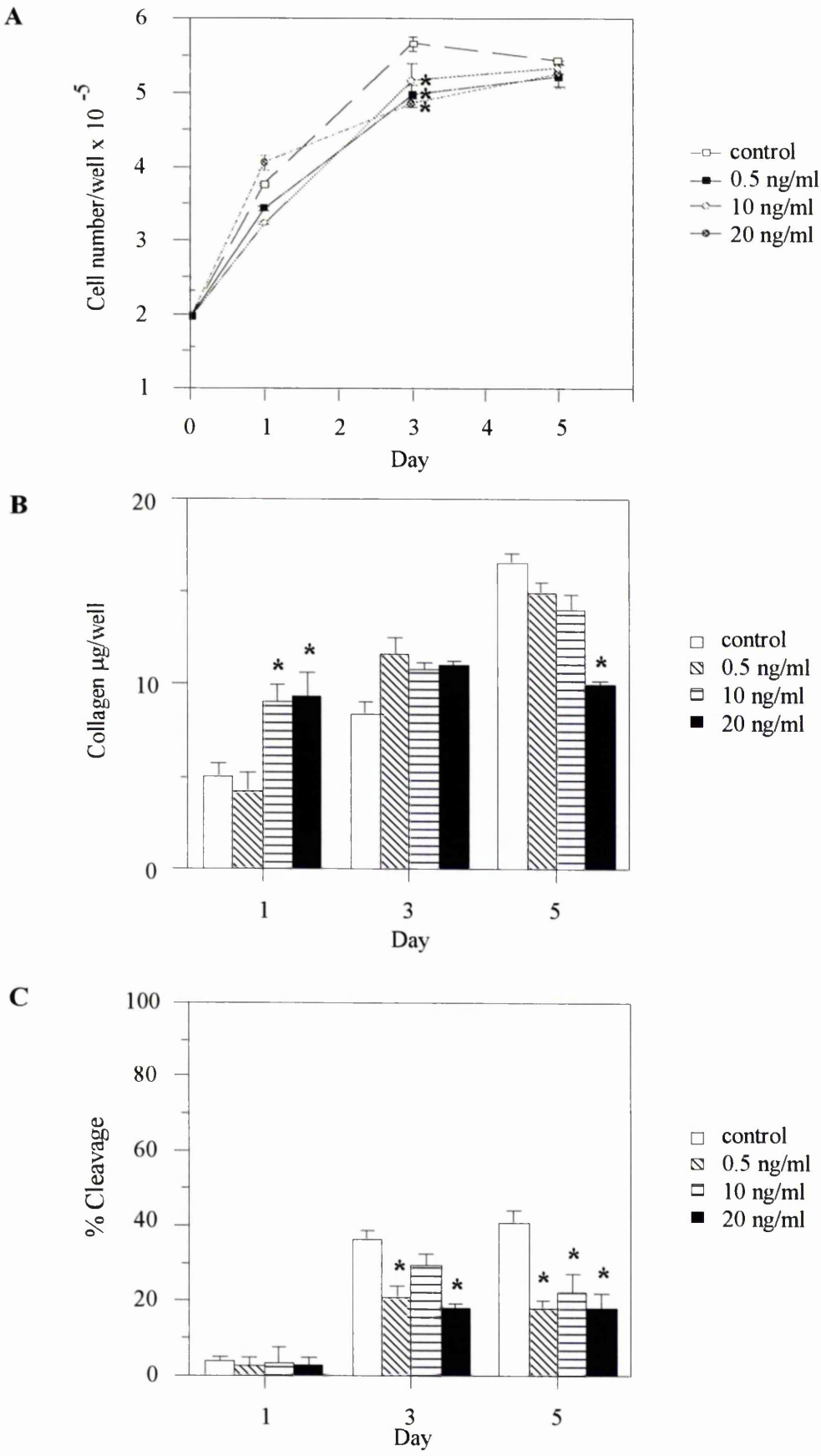
4.3, A). Collagen levels were significantly raised at day 1 with 10 and 20 ng/ml doses (80-85% increments), but by day 5 a dose dependent reduction of collagen, compared to the control was apparent (Figure 4.3, B). With the level of collagen in the 20 ng/ml treated cells significantly less than the control. This could have arisen from lower cell numbers at day 3, and collagen deposition may not have been detectable until day 5. Collagen in the cell layer could have been degraded by collagenase and other matrix degrading enzymes that are known to be stimulated by IL-1β (Mauviel, 1993), although the enzymes were not measured. Significantly less activity (Figure 4.3, C) was present in media from cells treated with IL-1β (0.5 and 20 ng/ml at day 3 and all doses at day 5) and is possibly the reason for the decreased levels of deposited collagen.

4.3.2 Effects of Interleukin-4

IL-4 is a pleiotropic cytokine expressed mainly by T lymphocytes and mast cells, with multiple immunomodulatory functions on a variety of cell types including fibroblasts, T cells, macrophages and osteoblasts (Banchereau & Rybax, 1994). During the early stages of fibrosis these inflammatory cells infiltrate tissue and may secrete IL-4 which is thought to be involved in connective tissue activation. In human osteoblast like cells IL-4 increases the expression of the proα1(VI) collagen gene mediating accumulation of hydroxyproline in the cell layer (Ishibashi *et al.*, 1995), and it has also been shown to stimulate proα1(I) collagen gene expression at the pretranslational level with

Figure 4.3 Effects of IL-1β on Cell Growth, Collagen Synthesis and C-proteinase Activity

MG63 cells were cultured, in 6 well plates, for 24 hr in FCS-supplemented media, washed and grown in Nutridoma supplemented DMEM media, supplemented with Il-1β. ***Legends***, indicate concentrations Il-1β of added. The group number per time point was 3. ***A***, Cell number was determined using the MTT assay. ***B***, Insoluble collagen laid down in the cell layer was determined using the Sircol assay. ***C***, C-proteinase activity is expressed as % cleavage, where 89 μl of media from treated cells was added to 1 μg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating) under reducing conditions, fluorography, and scanning densitometry. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).


A
Cell number/well x 10 -5
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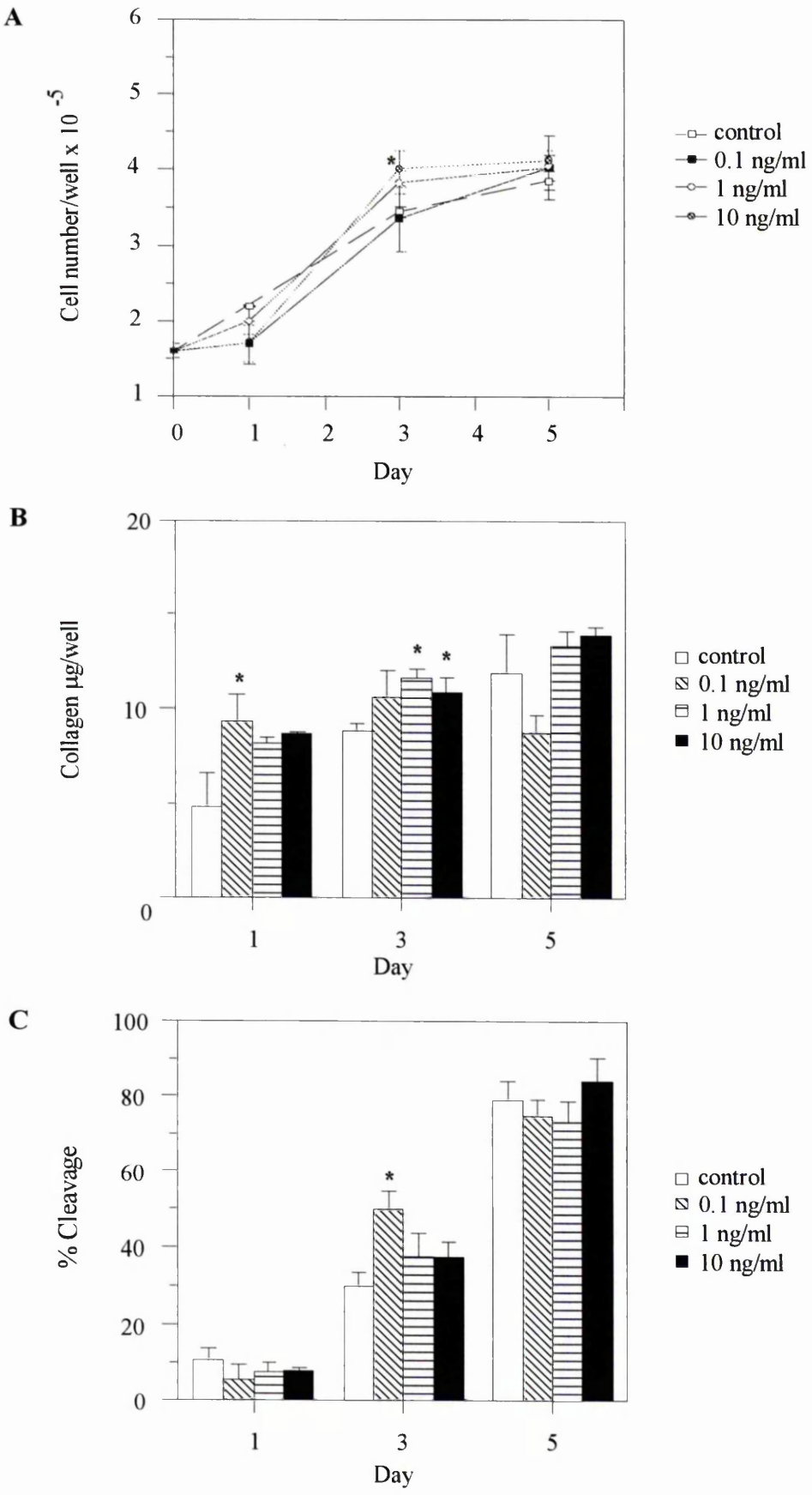
control
0.5 ng/ml
10 ng/ml
20 ng/ml
B
Collagen µg/well
Day
control
0.5 ng/ml
10 ng/ml
20 ng/ml
C
% Cleavage
Day
control
0.5 ng/ml
10 ng/ml
20 ng/ml

physiological concentrations of 10 ng/ml in human fibroblast monolayer cultures (Gillery *et al.*, 1992).

Doses based around the physiological concentration (0.1, 1 and 10 ng/ml) of human recombinant IL-4 were added to MG63 cells, and cell proliferation, collagen and C-proteinase activity determined over 5 days (Figure 4.4). Initially, cells cultured in the presence of IL-4 had prolonged cell cycles, and grew slower than the control (Figure 4.4, A), but by day 3, 10 ng/ml treated cells had responded with significantly faster proliferation rates. At day 5, cell growth was constant at ~4 x 10^5 cells/well, as confluency was reached. The collagen levels (Figure 4.4, B) in the cell layer of 0.1 ng/ml treated cells (9.28 μg) at day 1, was significantly greater than the control (4.8 μg) and, with all other doses, a trend towards an increase (70-95%) was observed. Previously, an increase of 60-100% had been observed in the fibroblast monolayers (Gillery *et al.*, 1992). This increase in collagen was not related to cell number, as no difference was observed at that time point (day 1). Collagen levels at day 5, were reduced by 17% in cells cultured in the presence of 0.1 ng/ml of IL-4 compared to levels at day 3, this could have arisen from degradative enzymes, such as collagenase or could have been artefactual. Significant increase in activity of C-proteinase (Figure 4.4, C) was only seen at day 3 (0.1 ng/ml treated cells). At all other times no significant difference compared to the controls were observed.

Figure 4.4 Effects of IL-4 on Cell Growth, Collagen Synthesis and C-proteinase Activity

MG63 cells were cultured, in 6 well plates, for 24 hr in FCS-supplemented media, washed and grown in Nutridoma supplemented DMEM media, supplemented with IL-4. ***Legends***, indicate concentrations Il-4 of added. The group number per time point was 3. ***A***, Cell number was determined using the MTT assay. ***B***, Insoluble collagen laid down in the cell layer was determined using the Sircol assay. *C*, C-proteinase activity is expressed as % cleavage, where 89 µl of media from treated cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating) under reducing conditions, fluorography, and scanning densitometry. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).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Cell number/well x 10 -5
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1
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1
2
3
4
5
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml
B
Collagen µg/well
20
10
0
1
3
5
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml
C
% Cleavage
100
80
60
40
20
0
1
3
5
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml

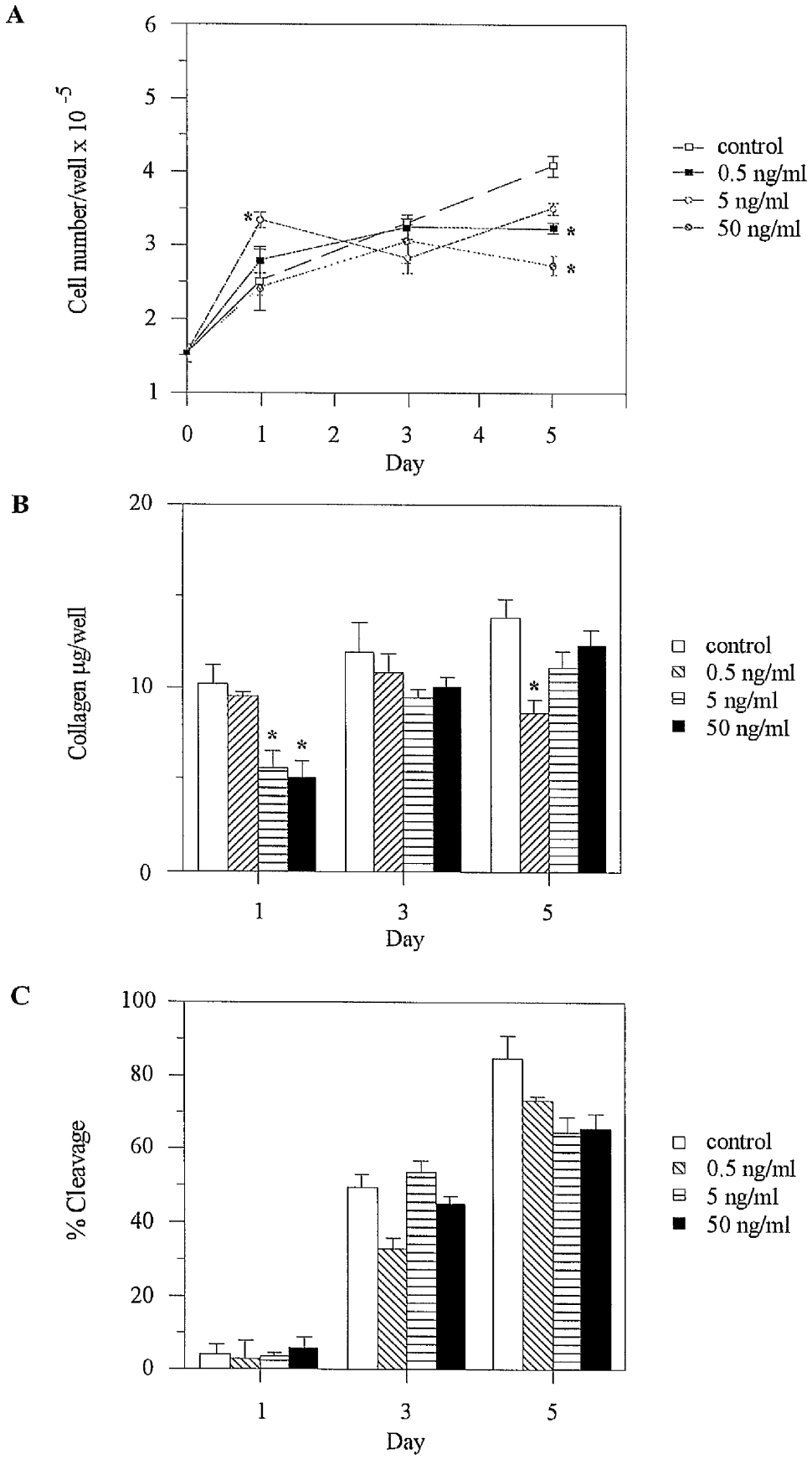
4.3.3 Effects of Epidermal Growth Factor

EGF, a polypeptide synthesised by various cells including platelets, keratinocytes and activated macrophages, has a wide variety of biological functions. The major activity is to promote proliferation and differentiation of mesenchymal and epithelial cells via specific transmembrane receptors (Chen *et al.*, 1987). It has a significant role in the development of keloid scars (Kikuchi, 1995), and has therapeutic potential as an accelerator to wound healing (Schultz *et al.*, 1991). Binding of EGF to its receptor causes autophosphorylation and subsequent activation of the cellular tyrosine kinases, and arachidonic acid pathways, is essential for rapid changes in intracellular calcium levels, activation of gene expression and a number of other diverse effects (Chen *et al.*, 1987). EGF *in vitro* stimulates collagenase and stromelysin at the pretranslational level in normal skin fibroblasts (25 ng/ml), and is accompanied with a decrease in procollagen mRNA. Growth of MG63 cells was stimulated in a dose dependent manner by EGF, an effect completely inhibited by TGFβ1, which was shown to reduce tyrosine kinase activities by decreasing tyrosine phosphorylation of the receptor (Mioh & Chen, 1989).

MG63 cells grown in the presence of 5 ng/ml EGF (Figure 4.5, A) showed significantly greater initial growth than the control. By days 3 and 5 cell cycles became prolonged at all doses, and by day 5, control cells had proliferated at a significantly greater rate than cells treated with 0.5 and 50 ng/ml EGF. Cells grown in the presence of 5 and 50 ng/ml EGF had a cell layer with significantly lower levels of collagen on day 1. By day 5 collagen levels were still lower in treated cells than in the control, significantly in the

Figure 4.5 Effects of EGF on Cell Growth, Collagen Synthesis and C-proteinase Activity

MG63 cells were cultured, in 6 well plates, for 24 hr in FCS-supplemented media, washed and grown in Nutridoma supplemented DMEM media, supplemented with EGF. ***Legends***, indicate concentrations EGF of added. The group number per time point was 3. ***A***, Cell number was determined using the MTT assay. ***B***, Insoluble collagen laid down in the cell layer was determined using the Sircol assay. ***C***, C-proteinase activity is expressed as % cleavage, where 89 µl of media from treated cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating) under reducing conditions, fluorography, and scanning densitometry. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).


A
Cell number/well x 10 -5
6
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2
1
0
1
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3
4
5
Day
control
0.5 ng/ml
5 ng/ml
50 ng/ml
B
Collagen µg/well
20
10
0
1
3
5
Day
control
0.5 ng/ml
5 ng/ml
50 ng/ml
C
% Cleavage
100
80
60
40
20
0
1
3
5
Day
control
0.5 ng/ml
5 ng/ml
50 ng/ml

case of cells treated with 0.5 ng/ml EGF.

At day 3 and 5, EGF stimulated cells were secreting less C-proteinase into the media than the control, although the levels were not statistically significant (Figure 4.5, C). From the autoradiograph of the electrophoretic assay (Figure 4.6, lanes 5-8), the presence of α1(I) and α2(I) chains indicate N-proteinase activity in the control. EGF has slightly inhibited activity or synthesis of both C and N-proteinase. The low levels of collagen (Figure 4.5, B) were correlated to those of C-proteinase (day 3 and 5) (Figure 4.5, C).

4.3.4 Effects of Fibroblast Growth Factor basic

FGFb is a polypeptide growth factor, that is a potent regulator of cell proliferation, differentiation and function (Baird & Bohlen, 1990). FGFb acts on all cells of mesodermal origin and many others, including fibroblasts, endothelial cells, osteoblasts and smooth muscle cells. Examples of function include roles in modulating angiogenesis, wound healing and tissue repair, embryonic development and differentiation, neural degeneration, and haematopoiesis (Gospodarowicz *et al.*, 1986). As a potent mitogen, it triggers proliferation at concentrations as low as 5 pg/ml, but at the same time stabilises the phenotypic expression of cultured cells.

FGFb was used to stimulate cells at 0.1, 1 and 10 ng/ml and the effects on proliferation, collagen and C-proteinase measured are shown in figure 4.7. By day 1 a significant increase in proliferation of 100%, 112% and 119% was obtained at 0.1, 1

EGF was added to cells, in Nutridoma supplemented DMEM, 24 hr after seeding and incubated for 1, 3 and 5 days. Media (89 µl) from experimental cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, fluorography, and scanning densitometry. ***Lane C***, 1 µg CET-procollagen unincubated; ***lane 4C***, 1 µg CET-procollagen incubated for 4 hr; ***lanes 1, 5 and 9*** media from untreated cells; ***lanes 2, 6 and 10*** media from cells treated with 0.5 ng/ml; ***lanes 3, 7 and 11*** media from cells treated with 5 ng/ml; ***lanes 4, 8 and 12*** media from cells treated with 50 ng/ml. The sample from lane 3 was lost due to laboratory accident.

Figure 4.6 C-proteinase Activity in MG63 Cells Treated with EGF for up to 5 Days in Culture

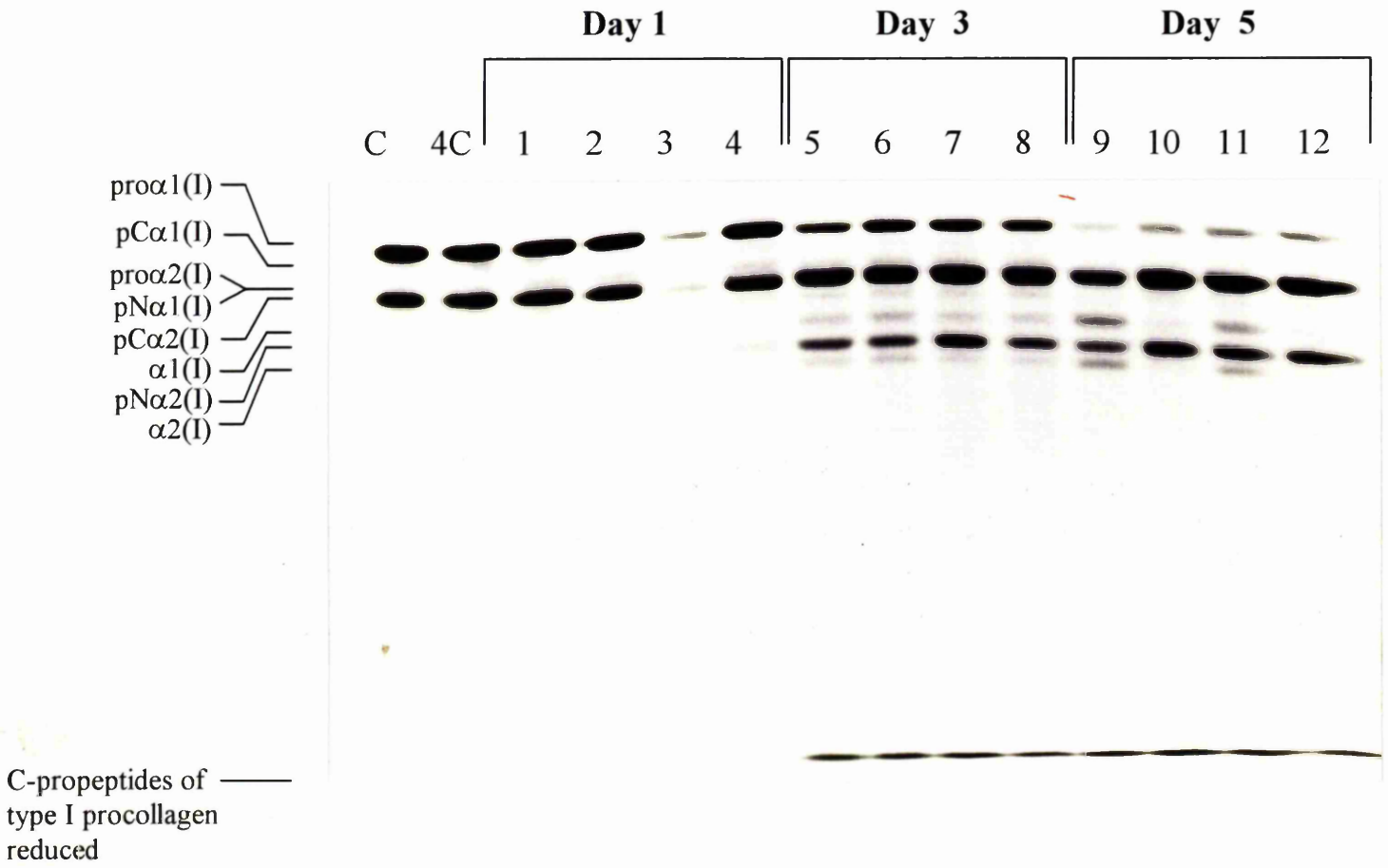
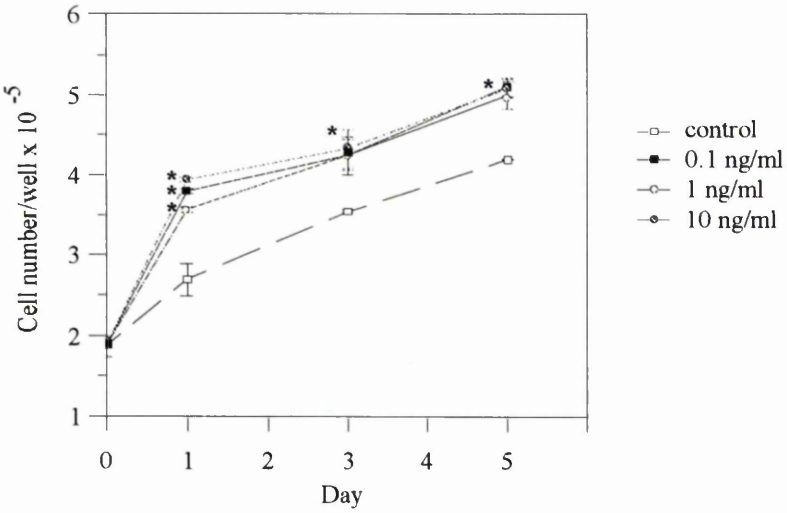


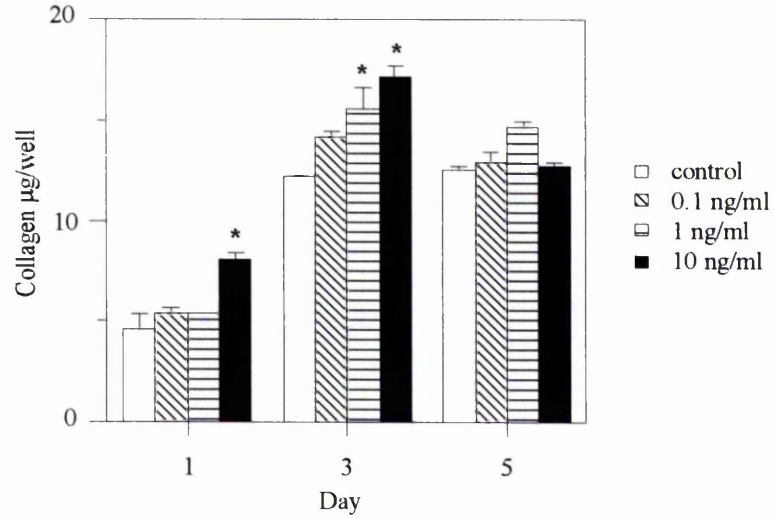
Figure 4.7 Effects of FGF on Cell Growth, Collagen Synthesis and C-proteinase Activity

MG63 cells were cultured, in 6 well plates, for 24 hr in FCS-supplemented media, washed and grown in Nutridoma supplemented DMEM media, supplemented with FGF. ***Legends***, indicate concentrations FGF of added. The group number per time point was 3. ***A***, Cell number was determined using the MTT assay. ***B***, Insoluble collagen laid down in the cell layer was determined using the Sircol assay. ***C***, C-proteinase activity is expressed as % cleavage, where 89 µl of media from treated cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating) under reducing conditions, fluorography, and scanning densitometry. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).

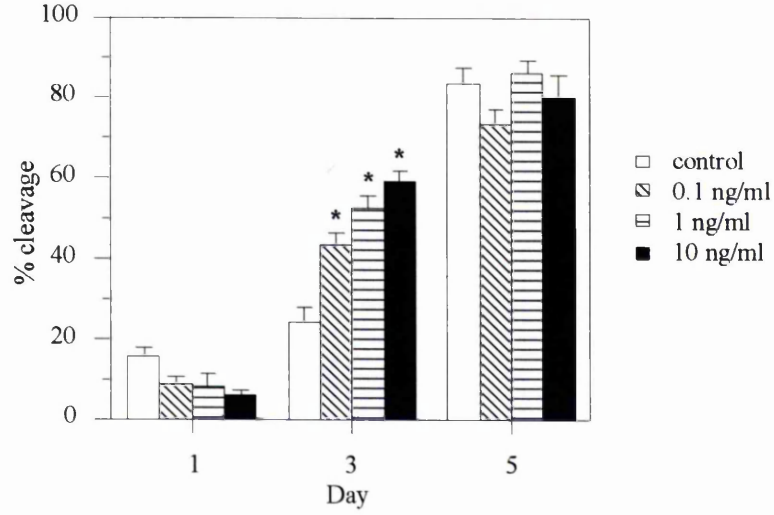
A


Cell number/well x 10 -5
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml

B


Collagen µg/well
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml

C

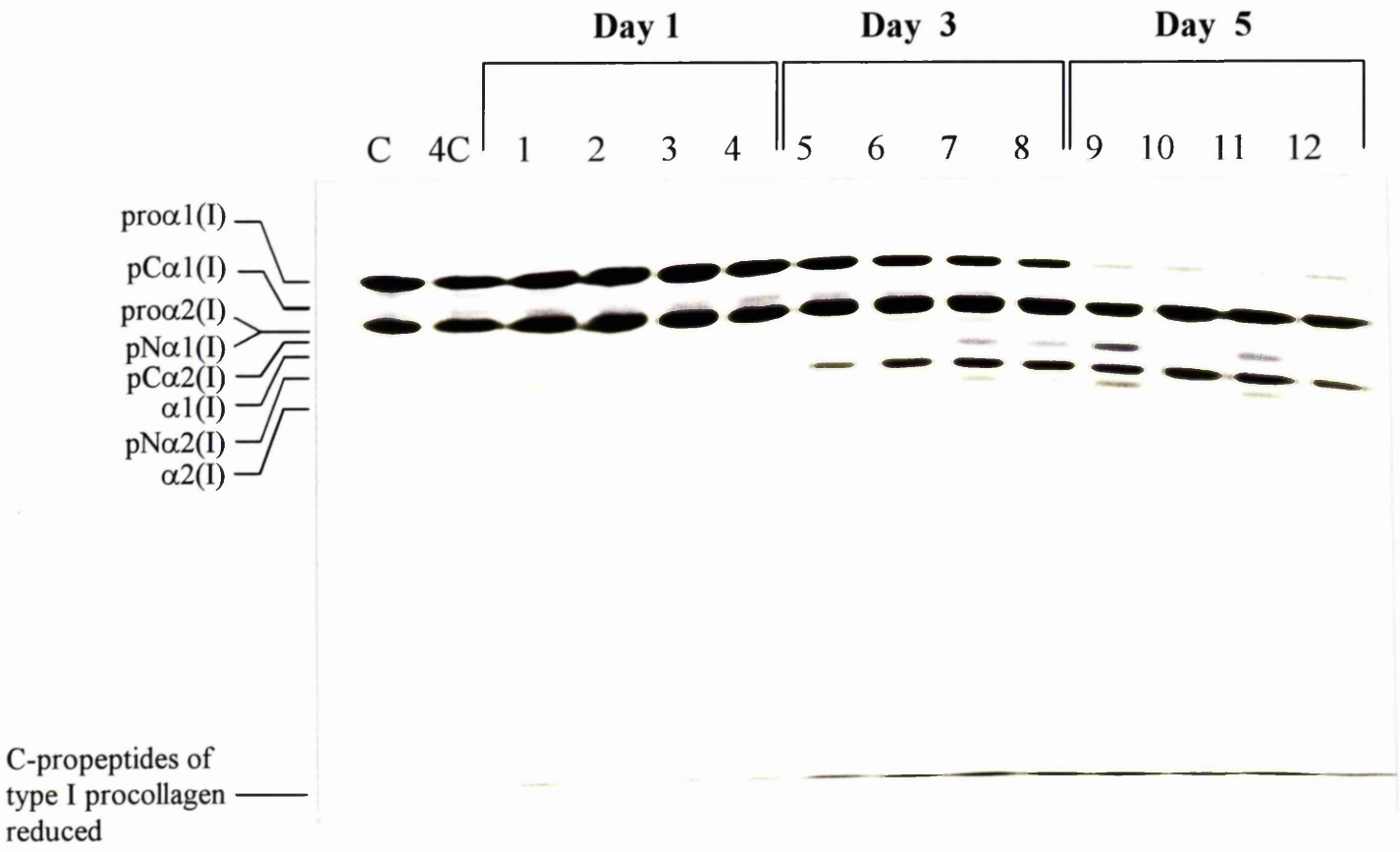

% cleavage
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml

and 10 ng/ml FGFb respectively (Figure 4.7, A). FGFb was a potent mitogen for the MG63 cells, throughout the 5 days incubation. A significant increase (1.6 fold) in collagen deposition was observed, after 24 hr incubation with 10 ng/ml FGFb (Figure 4.7, B). However, incubation with 0.1 and 1 ng/ml FGFb did not alter the collagen production. By the third day collagen deposition was increased in control cells and by 34%, 31% and 47% in cells incubated in the presence of 0.1, 1 and 10 ng/ml FGFb respectively, relative to collagen levels on day 1. The effect increased in a dose dependent manner and was significantly different from control values at 1 and 10 ng/ml. On day 5, the collagen in the cell layer in the stimulated cultures was not significantly different from control cultures. The absolute levels in the control cultures was equivalent to that determined on day 3, but surprisingly, the levels in the FGFb stimulated cells had dropped at all three concentrations (Figure 4.7, B). At 10 ng/ml FGFb the decrease was from 17μg to 12 μg collagen. This could have been due to degradation caused by upregulated collagenase and stomelysin (Mauviel, 1993).

At day 3, C-proteinase activity, expressed as % cleavage, increased significantly by 95%, 136%, 172% at 0.1, 1, and 10 ng/ml FGFb, respectively, relative to day 3 control cell, in a dose dependent response to FGFb (Figure 4.7, C). Increased activity at day 3 correlated with the increased levels of collagen (Figure 4.7, B). In the autoradiograph (Figure 4.8), collagen α1(I) and α2(I) chains were clearly observed in the cells stimulated with 1 and 10 ng/ml treated cells at day 3 (Figure 4.8, lanes 7 and 8). The prescence of α1 and α2 indicates the prescence of N-proteinase as well as the C-proteinase. At day 5 (Figure 4.7, C) over 70% of the procollagen cleavage in the assay incubation had occurred, with the disappearance of the proα1(I) chain being

FGFb was added to cells, in Nutridoma supplemented DMEM, 24 hr after seeding and incubated for 1, 3 and 5 days. Media (89 µl) from experimental cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, fluorography, and scanning densitometry. ***Lane C***, 1 µg CET-procollagen unincubated; ***lane 4C***, 1 µg CET-procollagen incubated for 4 hr; ***lanes 1, 5 and 9*** media from untreated cells; ***lanes 2, 6 and 10*** media from cells treated with 0.1 ng/ml; ***lanes 3, 7 and 11*** media from cells treated with 1 ng/ml; ***lanes 4, 8 and 12*** media from cells treated with 10 ng/ml.

Figure 4.8 C-proteinase Activity in MG63 Cells Treated with FGFb for up to 5 Days in Culture



prominent. There were no significant differences between the treated cells and the control. The control media at day 5, (Figure 4.8, lane 9) had elevated levels of N-proteinase compared to media from FGFb stimulated cells, this may have been due to N-proteinase being switched on when there was an abundance of pNα1(I) and pNα2(I). As the levels of collagen fell over the last 48 hr (Figure 4.8, B) this would suggest that degradative enzymes, possibly, MMPs were solubilising the collagen.

4.3.5 Effects of Transforming Growth Factor β

TGFβ1 is a polypeptide growth factor with a wide range of actions on most cells, as most have TGFβ1 receptors. Effects on cell proliferation, and differentiation may be stimulatory or inhibitory and is dependent on cell type, growth conditions, state of cell differentiation and the presence of other growth factors (for review Sporn *et al.*, 1986). TGFβ1 also regulates actions of other peptide growth factors, such as inhibiting the proliferation of EGF stimulated cells (Mioh & Chen, 1989). TGFβ1 mediates the stimulation of the ECM and inhibits subsequent degradation, illustrated by a 6 fold increase in type I collagen production in osteoblast-enriched cultures of foetal rat bone (Centrella *et al.*, 1992). One of the largest sources of TGFβ1 is found in bone, where it co-ordinates bone remodelling by stimulating osteoblasts and inhibiting osteoclasts, and it accelerates osteogenic differentiation during embryogenesis and tissue repair (Mizuno & Kuboki, 1995).

The effects of TGFβ1 at 0.1, 1, and 10 ng/ml on MG63 cell proliferation, collagen deposition in the cell layer and C-proteinase activity were monitored (Figure 4.9). In

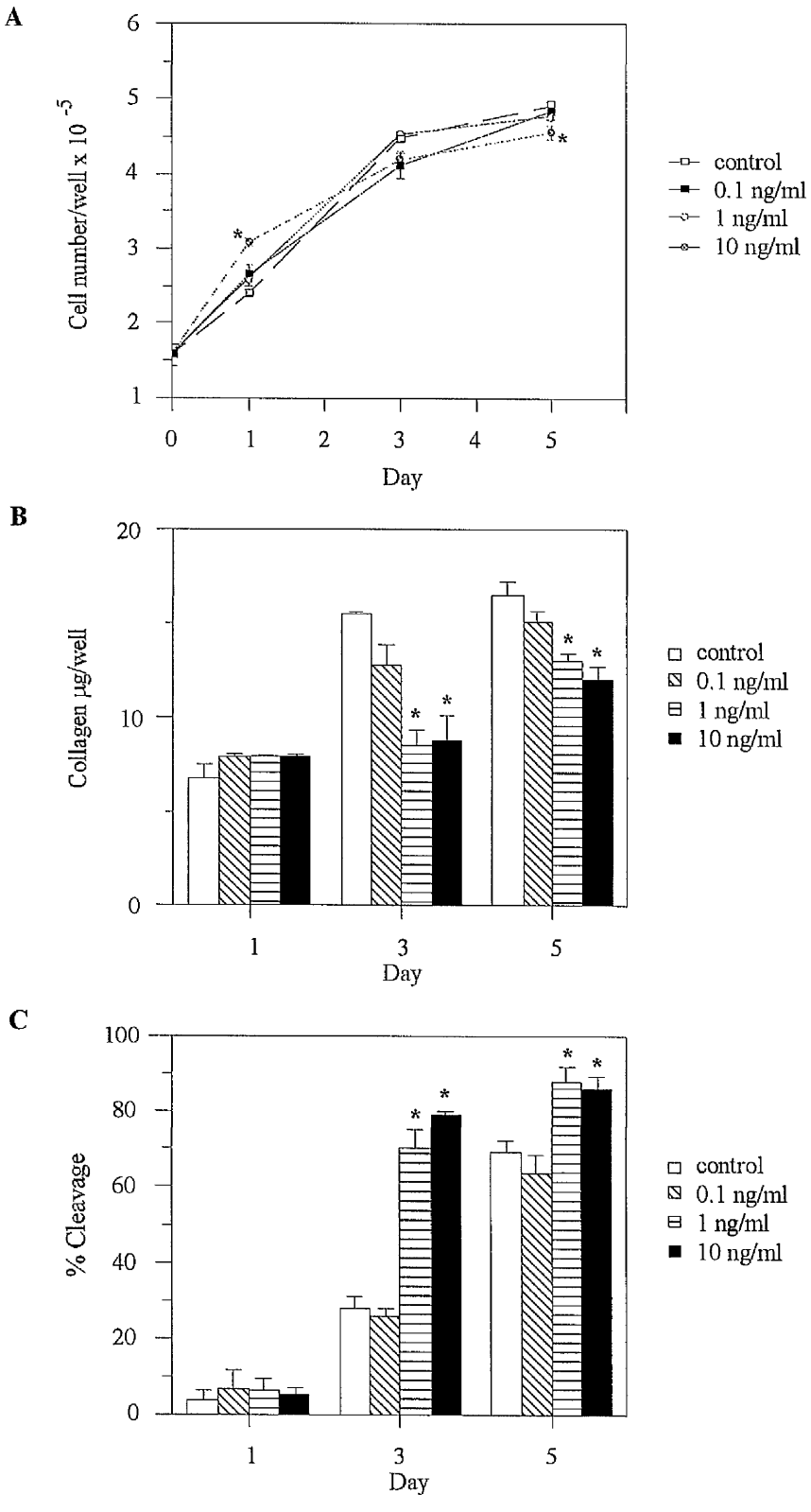
previous work, prolonged MG63 cell cycling with TGFβ1 was observed (Mioh & Chen, 1989), however this was not seen in this study. Initially a significant increase (27% of control) in cell number was apparent with cells cultured with 10 ng/ml TGFβ1, but not with cells incubated with 0.1 and 1 ng/ml (Figure 4.9, A). By day 5, 10 ng/ml TGFβ1 treated cells were growing at significantly slower rate than the control, and the 0.1 and 1 ng/ml treated cells were growing at a slower but not statistically different rate.

On day 1 TGFβ1 had no effect on collagen deposition (Figure 4.9, B). By day 3 collagen deposition had increased from 6.8 to 15.4 μg collagen in control cells and 7.9 to 12.4 μg collagen in the presence of 0.1 ng/ml TGFβ1. The collagen deposited by cells grown in the presence of 0.1 and 10 ng/ml was significantly less than the control (47 and 44% respectively) and had not increased from day 1. The collagen had increased to 15.8, 15, 12.5 and 11.9 μg/well on day 5 at 0, 0.1, 1 and 10 ng/ml TFGβ, and in the case of the higher two doses was significantly decreased relative to control on day 5.

The collagen deposition data does not correlate with the C-proteinase activity in media taken from the cells (Figure 4.9, C). As on both days 3 and 5 decreased collagen deposition is observed in cultures with the most C-proteinase activity. C-proteinase

Figure 4.9 Effects of TGFβ1 on Cell Growth, Collagen Synthesis and C-proteinase Activity

MG63 cells were cultured, in 6 well plates, for 24 hr in FCS-supplemented media, washed and grown in Nutridoma supplemented DMEM media, supplemented with TGFβ1. ***Legends***, indicate concentrations TGFβ1 of added. The group number per time point was 3. ***A***, Cell number was determined using the MTT assay. ***B***, Insoluble collagen laid down in the cell layer was determined using the Sircol assay. ***C***, C-proteinase activity is expressed as % cleavage, where 89 µl of media from treated cells was added to 1 µg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating) under reducing conditions, fluorography, and scanning densitometry. Statistical significance (*, $p<0.025$) was determined using the Student's t test for treated cells relative to the control. Error bars indicate standard error of mean (SEM).


A
Cell number/well x 10 -5
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml
B
Collagen µg/well
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml
C
% Cleavage
Day
control
0.1 ng/ml
1 ng/ml
10 ng/ml

was low in the control and in TGFβ1 treated cultures on day 1. On day 3 enzyme activity in the medium had increased 7 fold in control. In the cells incubated in the presence of 1 and 10 ng/ml TGFβ1 the enzyme levels were significantly higher than in control cultures; 166 and 185% respectively, compared to control. On day 5 C-proteinase activity in control cells and in cells cultured with 0.1 ng/ml TGFβ1 had increased activity compared to that seen on day 3, however, 1 and 10 ng/ml TGFβ1 still caused an increase of C-proteinase activity over that in control cultures.

On days 3 and 5 the assay gel of the media for C-proteinase activity shows that pCα1(I), α1(I) and α2(I) chains were apparent (Figure 4.10, lanes 7, 8, 11 and 12), suggesting that N-proteinase was cleaving both procollagen and the pNα1(I) and pNα2(I) chains. By day 5, the partially cleaved fragments, seen between the proα2(I) and the α1(I) chains, were finally cleaved or degraded, resulting in 90% conversion of procollagen into collagen monomers (Figure 4.10, lanes 11 and 12). It is possible that the failure to deposit increased collagen in this cell line is a result of decreased production of procollagen substrate for C- and N-proteinase.

4.3.5.1 Effects of inhibitors on enzyme activity in media from TGFβ1 stimulated cells

The presence of partially cleaved fragments in the SDS-PAGE assay autoradiogram, indicated the presence of non-specific cleavage, or specific cleavage of other C-proteinases. To examine further the identity of enzymes involved in cleaving procollagen in the media from TGFβ1 treated samples, the day 3 samples from the

TGFβ1 was added to cells, in Nutridoma supplemented DMEM, 24 hr after seeding and incubated for 1, 3 and 5 days. Media (89 μl) from experimental cells was added to 1 μg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, fluorography, and scanning densitometry ***Lane C***, 1 μg CET-procollagen unincubated; ***lane 4C***, 1 μg CET-procollagen incubated for 4 hr; ***lane +C***, 1 μg CET-procollagen and 0.5 units of human C-proteinase incubated for 4 hr; ***lanes 1, 5 and 9*** media from untreated cells; ***lanes 2, 6 and 10,*** media from cells treated with 0.1 ng/ml; ***lanes 3, 7 and 11,*** media from cells treated with 1 ng/ml; ***lanes 4, 8 and 12***, media from cells treated with 10 ng/ml.

Figure 4.10 C-proteinase Activity in MG63 Cells Treated with TGFβ1 for up to 5 Days in Culture

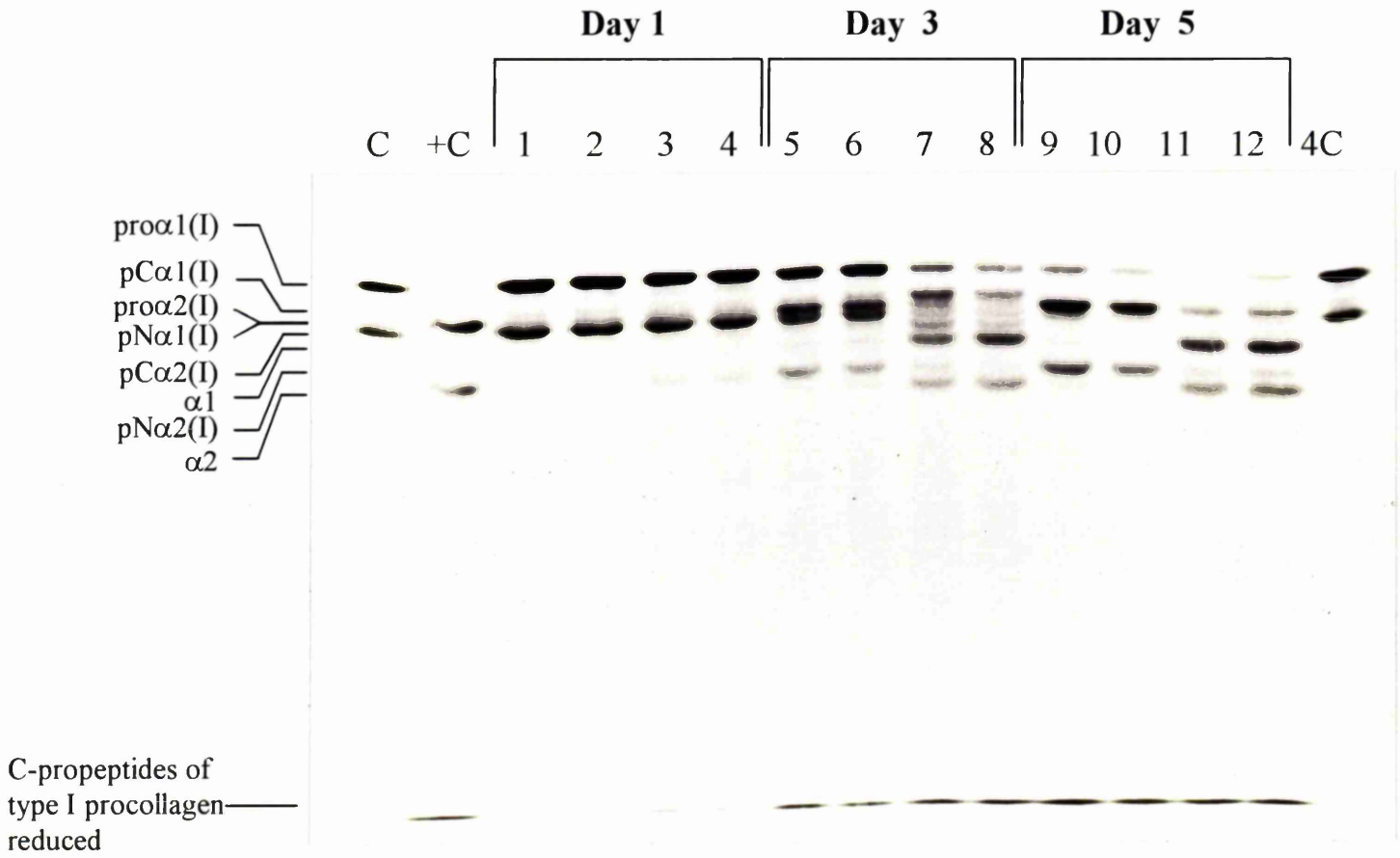
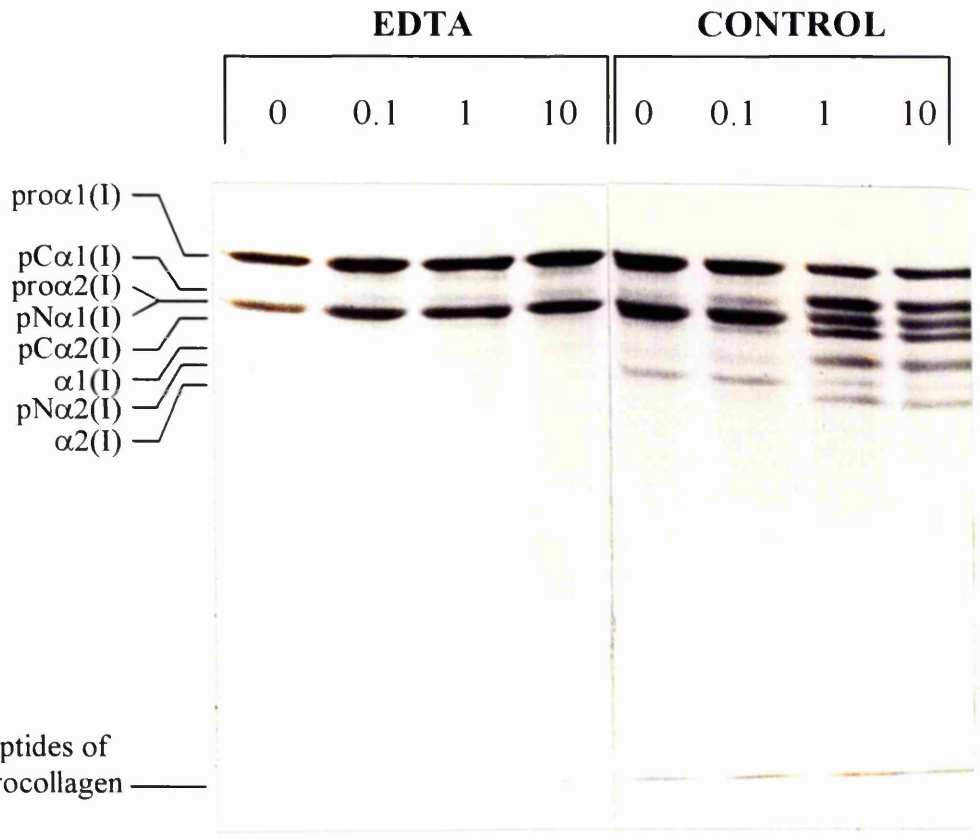


Figure 4.11 Effects of EDTA C- and N- proteinase Activity in Media from TGFβ1 Treated Cells



Samples were analysed by SDS-PAGE (7% separating), under reducing conditions, fluorography and scanning densitometry. Samples of media from TGFβ1 treated MG63 cells (day 3, at 0, 0.1, 1 and 10 ng/ml TGFβ1, from figure 4.10) were added to 10 mM ***EDTA*** and 1μg CET-procollagen with 0.02% (w/v) DS, and incubated for 4 hr at 37 °C. ***Lanes 0***, ***0.1***, ***1*** and ***10*** indicate the concentration of TGFβ1 at day 3. EDTA completely inhibited cleavage.

previous experiment (Figures 4.9 and 4.10) were analysed. Samples of media were incubated with and without the metal chelator EDTA (Figure 4.11). EDTA at 10 mM inhibited cleavage at all doses by 100%, again confirming that C-proteinase and the enzymes responsible for non-specific cleavage are metalloproteases.

4.4 DISCUSSION

Major problems had arisen with obtaining enough active enzyme, from which to obtain an N terminal sequence, so, in an attempt to increase levels in cell culture the effects of cytokine and growth factors on C-proteinase activity in MG63 cells was investigated. The effects of cytokines and growth factors on collagen and procollagen production is extensive, but little has been done to characterise the effects on C-proteinase and nothing specifically in MG63 cells. It was not known whether co-ordinated synthesis of C-proteinase and its substrate, procollagen occurs.

A cell culture system was set up and conditions optimised to enable the detection of C-proteinase in media after one day of incubation. Cells were seeded at 1.5×10^5/ well in Costar 6 well plates, in 1 ml of FCS-supplemented DMEM media containing ascorbate. After 24 hr, the media was removed, cells washed thoroughly, and DMEM containing Nutridoma and growth factor were added for a five day incubation period. The growth factors chosen, were known to significantly effect collagen synthesis *in vitro* and included FGFb and TGFβ1, which as potent mitogens and activators of fibroblasts, cause major increases in collagen, and overproduction of ECM in numerous progressive fibroproliferative diseases (Kovacs, 1991).

IL-1β stimulated an increase in collagen synthesis over that in control cultures during the early stages of incubation, which was gradually reversed by day 5. By days 3 and 5, C-proteinase levels increased over that observed on day 1 but were significantly lower than control levels, this could have been a result of IL-1β stimulating the expression of MMPs, interstitial collagenase, both 72 and 92 kDa gelatinases and stromelysin-1, at the pre-transcriptional level (Mauviel, 1993). IL-4, induced a variable and small increase in collagen levels prior to day 5, which was not mirrored by any consistent alterations in C-proteinase levels.

EGFs role in collagen synthesis is unclear, as it activates gene expression of collagenase and stromelysin (Mauviel, 1993), and reduces procollagen mRNA levels in MG63 cells, promoting proliferation and probably collagen synthesis (Mioh & Chen, 1989). In this study, EGF caused an initial burst of proliferation and a reciprocal effect on collagen synthesis. This is explained solely by the tyrosine kinase activity of the transmembrane EGF receptor, which is responsible for the subsequent, increased transcription of MMP genes and stimulatory effects on cell division (Chen *et al.*, 1987). As the initial increase in proliferation is not followed by an increase in C-proteinase, the effect of EGF on MMPs could in principle tip the balance in collagen turnover towards degradation, so any increase in C-proteinase would not be detected.

FGFb was mitogenic for the MG63 cells throughout the 5 day incubation, a result consistent with mitogenic effect on all mesenchymal cells (Kovacs, 1991). Consequently, by day 3, the levels of collagen and C-proteinase were significantly raised in a dose dependent manner. Both procollagen C- and N- proteinases were

upregulated, in direct correlation with an increase in deposited collagen. This effect is possibly due only to the increase in cell growth, and not a direct effect on C-proteinase. By day 5, however, no upregulation of C-proteinase was observed, and the collagen deposited in the cell layer returned to control values. FGFb may have increased the expression of MMPs which could account for the reduction in levels of deposited collagen. So if FGFb was used in mass cell culture it would not significantly increase the total quantity of C-proteinase available, it would just speed up the generation.

TGFβ1 had an interesting effect on MG63 cells. No significant and prolonged effect was seen on cell growth, so the effects on collagen and C-proteinase activity are independent to proliferation. By day 3 the levels of C- and N-proteinase were both significantly raised in the cultures stimulated with 1 and 10 ng/ml of TGFβ1. This stimulation was maintained for the five day incubation.

Remembering that BMP-1 has been identified as C-proteinase (Kessler *et al.*, 1996; Li *et al.*, 1996), it has been suggested that a product or products of the BMP-1 gene may interact and activate TGFβ-like molecules or TGFβ itself which then stimulate matrix production (Border & Ruoslahti, 1992). A possible theory is that alternatively spliced molecules from BMP-1, generating different C-proteinases, may have cleaved the procollagen precursor molecules, at slightly different sites to the -Ala-Asp- bond. TGFβ1 may also have upregulated extracellular enzyme synthesis, by inhibiting C- or N- natural inhibitors/MMPs, and, or, by increasing expression of enzyme at the pretranslational level.

Paradoxically, even though there has been an increase of C- and N-proteinase secretion into the growth media of these cells, on days 3 and 5 a decrease in deposited collagen is apparent at any day in cells cultured in the presence of 1 and 10 ng/ml TGFβ1. Another reason could be that TGFβ1 may have stimulated cytokines, such as IL-1β and EGF, which are subsequently involved in the stimulation of MMP expression. The decrease in collagen deposition and possible increase in collagenase expression are contrary to the expected results with TGFβ1 stimulated cells. The increase in C-proteinase production makes TGFβ1 a candidate for use in bulk cell culture for C-proteinase production, however, the actual effects of TGFβ1 on collagen would have to be determined prior to using the growth factor in this way.

Also an upregulation of N-proteinase was also apparent, in TGFβ1 stimulated cells, which may mask the specific activity of C-proteinase. Possible steps to separate the two proteinases, would be to use either a specific PP-Sepharose column, and or a lysyl-Sepharose column, as used in the purification of mouse C-proteinase and its enhancer (Kessler & Adar, 1989). Also the cost of adding a constant concentration of TGFβ1 to 20 flasks for 2-4 weeks, may limit its application. Results, from further investigations into the actual fold increases of C-proteinase over time, with numbers of cell flasks and cost being limiting parameters, would have to be known, prior to any mass MG63 cell culturing.

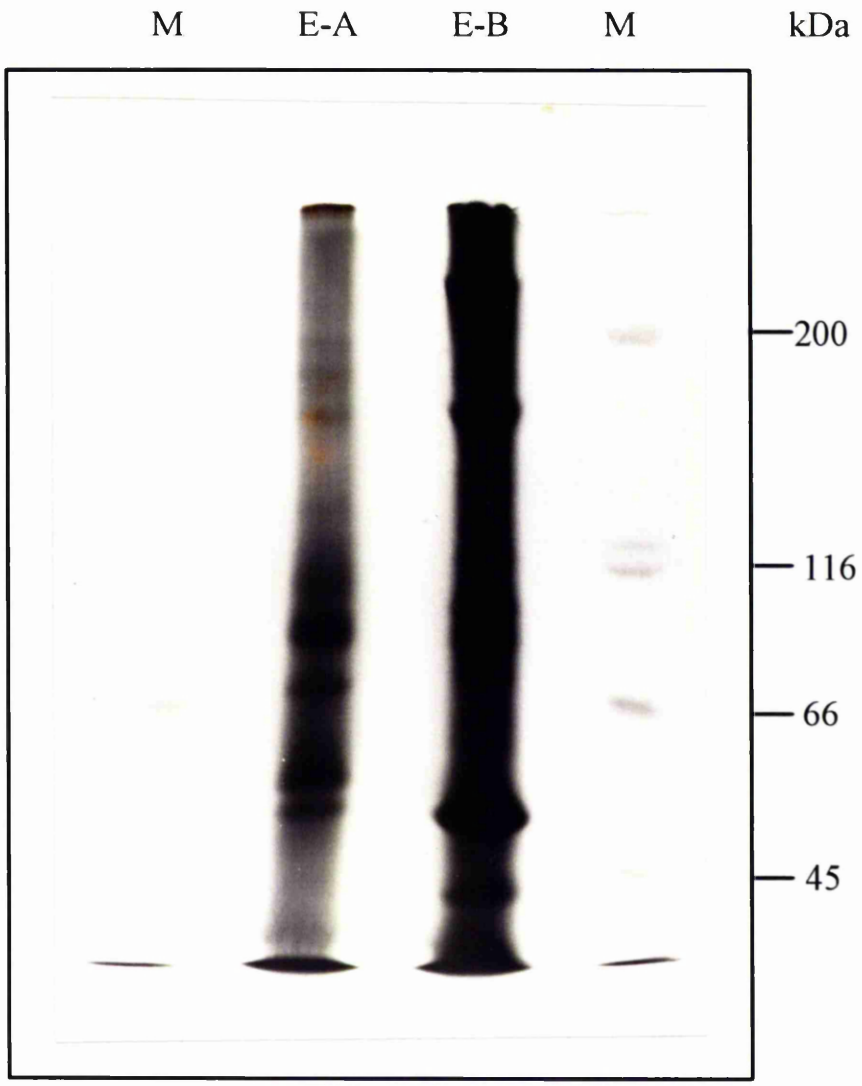
5. RESULTS PART III: HUMAN C-PROTEINASE IS BMP-1

5.1 TWO DIMENSIONAL ANALYSIS OF CNBR CLEAVAGE PRODUCTS

The final purification was dedicated to the analysis of the E-A and E-B samples by 2D CNBr cleavage mapping, to estimate the molecular weight of human C-proteinase, and to obtain partial N-terminal sequences. It was postulated that E-A and E-B are different forms of C-proteinase, possibly one is a precursor of the other, and so would have similar CNBr peptide patterns. If a protein in the E-A preparation, and one in the E-B preparation, both, had the same CNBr patterns, then the two proteins could be the human C-proteinase and sequences could then be obtained from cleaved peptides.

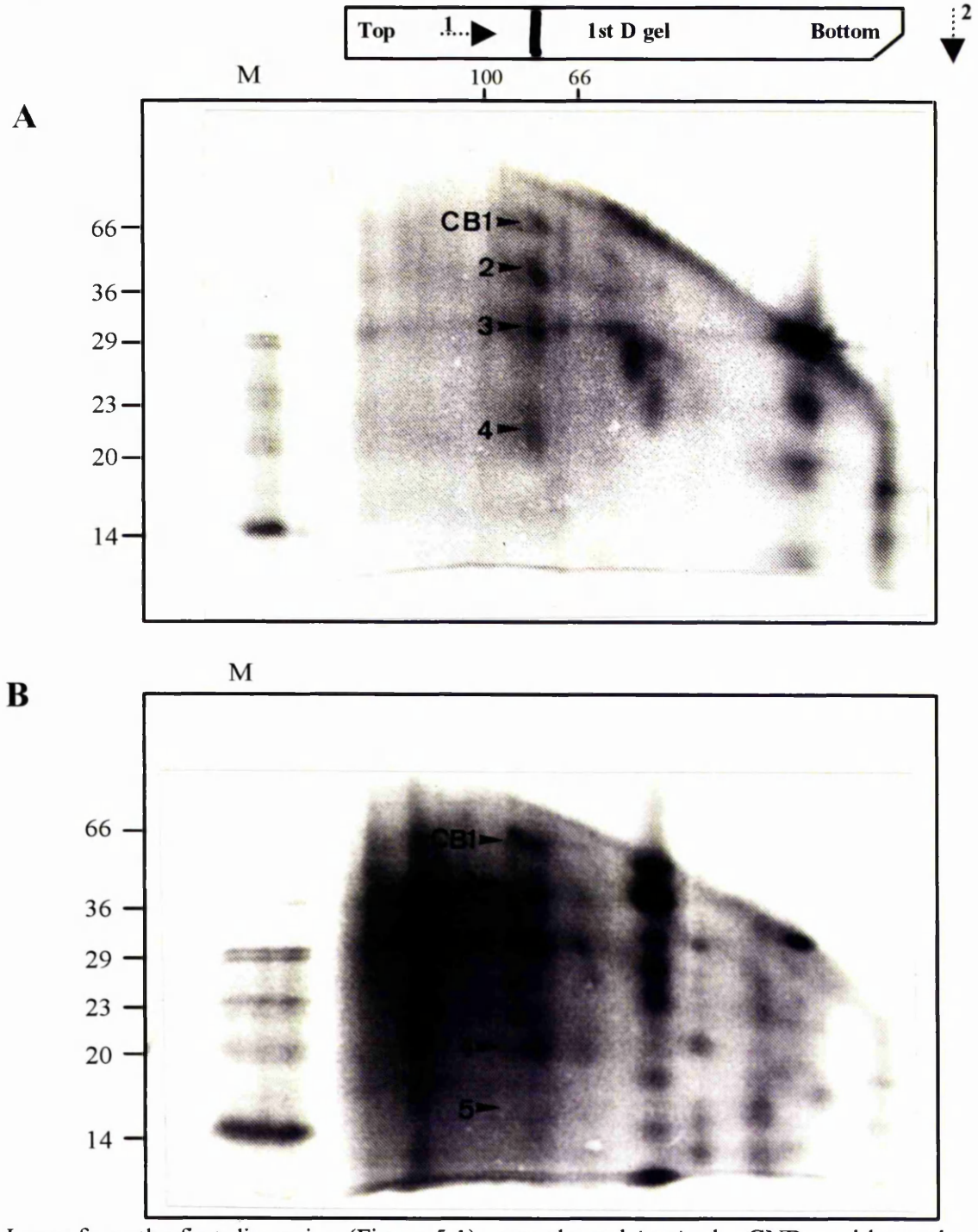
Proteins in E-A and E-B were radiolabelled (Figure 5.1) and from the autoradiograph of 40 µl samples 10-20 bands were seen in both. Lanes, on a 9.5% separating gel, containing separated radiolabelled proteins (200 µl sample), were carefully excised and placed into 70% formic acid. Proteins where then cleaved by immersing in a 50 mg/ml CNBr in 70% formic acid for 3 hr. The gel slice was then placed horizontally on to the top of a polymerised 15% separating gel. Stacking gel solution, applied around the lane, was allowed to polymerise and the gel run at 60 mA overnight. Proteins from the second dimension gel were blotted to PVDF, using a 25 mM Tris-HCl, 190 mM glycine in 10% (v/v) methanol transfer buffer, and then stained with Coomassie blue. Autoradiographs obtained from the blots showed a number of cleavage products (Figure 5.2).

Figure 5.1 Radiolabelled Human E-A and E-B



E-A and ***E-B*** (500 µl) from heparin-Sepharose chromatography (Figure 3.19) were radiolabelled with ^{14}C-formaldehyde, and 40 µl samples were analysed by SDS-PAGE (9.5% separating) under reducing conditions, and separated proteins visualised by fluorography.

Figure 5.2 Second Dimension Analysis of CNBr Cleavage Products of C-proteinase



Lanes from the first dimension (Figure 5.1) were cleaved *in situ* by CNBr and layered on to the second SDS-PAGE gel (15% separating) (schematic shows, by dotted arrow, the direction the first dimension (1) and the second dimension (2) gels were run in). Low molecular weight markers were used (***M,*** kDa), and the complete arrows show similar cleavage products, CB1-5, on ***A*** (E-A) and ***B*** (E-B), from a band at ~80 kDa.

5.1.1 Analysis of Cleavage Peptide Patterns

The only similarity between the E-A and E-B cleavage pattern was in 4/5 peptides both found from a protein band originating at 80 kDa. The experiment was repeated, and from a band of 80 kDa identical patterns were seen in both duplicated samples. With this duplicated result, the peptides from E-A and E-B (Figure 5.2), which had similar molecular weights (Table 5.1) were carefully excised from the blot and analysed by N-terminal sequencing.

Table 5-1 Molecular Weights of Peptides

	CB1	CB2	CB3	CB4	CB5
E-A	66.6	50.7	37.5	19.6	/
E-B	65	49.5	36	19	16

CB indicates CNBr cleaved peptides
Molecular weight (kDa) of cleaved peptides determined from low molecular weight markers in control lane

Unfortunately during N-terminal sequencing of all the peptides the background noise was too high, and the resultant signal too weak to allow residues to be identified. As the final human C-proteinase preparation was designated to this piece of work, there was insufficient material with which to repeat the experiment with an increased concentration of sample to increase the chance of obtaining N-terminal sequences.

5.2 DISCUSSION

From the cytokine and growth factor studies on C-proteinase activity, as discussed in results chapter II, FGFb and TGFβ1 both upregulated the synthesis of C-proteinase. However, the use of these two growth factors was probably not feasible, due to cost, in mass cell culture. Therefore, in a final attempt to obtain a N-terminal sequence of human C-proteinase, a whole purification, was dedicated to the analysis of E-A and E-B, by CNBr cleavage, and analysis by 2D SDS-PAGE.

All proteins in both E-A (4.6 mg protein, ~12μg C-proteinase) and E-B (6.2 mg protein, ~17 μg C-proteinase) samples were radiolabelled with ^{14}C-formaldehyde, after precipitation using trichloroacetic acid, and then split into two independent groups. The experiment was carried out in duplicate for both E-A and E-B samples.

On the first dimension gel, the, now identified 60 kDa enhancer molecule (Takahara *et al.*, 1994a) was possibly the observed 60 kDa protein in the E-B sample. Suggesting that the mouse C-proteinase, which is only active with the enhancer, purified by Kessler & Adar (Kessler & Adar, 1989) may be the chick E-B equivalent.

From the second dimension of CNBr treated samples, in duplicate, and in both E-A and E-B samples, the same CNBr peptide cleavage pattern was observed, all from an originating ~80 kDa protein. From observing this result I was confident enough to suggest that these were products from the same gene, possibly the human C-proteinase gene. However, no N-terminal sequences could be obtained from any of the peptides, due to high background signals. If time had allowed, another purification would have

been carried out, and the experiment repeated by dedicating the whole enzyme to one run, thereby, concentrating up the protein, to obtain better N-terminal sequence signals.

Two months after completing this final part of my studies, procollagen C-proteinase was identified, by two independent research groups, as BMP-1 (Kessler *et al.*, 1996; Li *et al.*, 1996). BMP-1, is a 80 kDa N-glycosylated metalloprotease, capable of inducing cartilage and bone formation (Reddi & Huggins, 1981). It was first co-purified along with the TGFβ-related BMPs 2A, 3, and 4 from bovine bone extract (Wozney *et al.*, 1988). It is characterised by three CUB domains and one EGF-like domain, which are both involved in protein-protein interactions, and the EGF probably confers calcium dependency on the molecule (Handford *et al.*, 1990). As the protein is an N-glycosylated, metalloprotease, found in bone, it links in with my studies in purifying a C-proteinase from an osteosarcoma cell line, where the overproduction of collagen in the bone cancer, may be linked to alternatively spliced BMP-1 transcripts (Takahara *et al.*, 1994b), which may have C-proteinase activity.

6. GENERAL DISCUSSION

GENERAL DISCUSSION

The skeletal integrity of animals is dependent upon the architecture and mechanical function of the variety of connective tissues that provide strength and rigidity. The same basic components are found in most connective tissues: collagen in the form of multimeric cross-linked fibrils, proteoglycans, glycoproteins, elastic fibres, a cellular component, water and minerals. The fibrillar collagens, types I, II, III, V and XI, which are the most abundant components, provide a framework for the attachment of a wide variety of connective tissue macromolecules. Defects in the structure or biosynthesis of fibrillar collagen are the cause of, or are associated with various acquired or genetic disorders that are characterised by symptoms such as brittle bones (osteogenesis imperfecta), or with pathologic fibrogenesis in diseases such as liver cirrhosis, pulmonary fibrosis and osteoarthritis.

Type I procollagen C-proteinase is central to the formation of collagen fibrils, and thus in progressive fibroproliferative disease. Firstly, it specifically cleaves the C-propeptide from the soluble pN-collagen molecules, initiating the assembly of insoluble fibrils, and, secondly, it activates the pro-lysyl oxidase enzyme, critical to cross-link formation which stabilises the triple helices.

The idea behind this study was to characterise human C-proteinase and in particular to obtain an amino acid sequence from purified enzyme, from which could be derived oligonucleotides primers which could be used to obtain a cDNA coding for C-proteinase. Secondly, to raise antibodies for studies such as *in situ* immunolocalisation

which could give an insight into the regulation and presence of C-proteinase in normal and diseased tissue. Subsequent to completing these studies, C-proteinase was identified as BMP-1. Therefore this thesis details the problems which arose in attempting to purify this low abundant C-proteinase, the identification, and partial purification, and investigation into the cytokine regulation of the synthesis of human C-proteinase from a novel source and finally, the relationship between this enzyme and BMP-1.

This study initially involved the partial purification of type I procollagen C-proteinase from chick embryo tendon, using a protocol devised by Hojima *et al.*, (Hojima *et al.*, 1985). As C-proteinase is present in tissue at extremely low levels, it was calculated that 300 doz chick embryos would be required to obtain enough C-proteinase, to obtain a N-terminal sequence. In an attempt to speed up purification, the dissection rate was doubled to 50 doz/week. However, contamination of 50% of cultures, actually slowed down the process, and the presence of bacterial proteases, in tendon cultures prior to those being obviously contaminated, probably degraded the sensitive C-proteinase, resulting in low yields. Eventually, the culturing protocol was optimised by splitting the yield of tendons and culturing them separately. Using this modification of the protocol two active forms, similar to that seen with chick purifications (purifications 4 and 5), were finally partially purified. However, many attempts at further purifying C-proteinase to homogeneity failed and the authentic material could not be identified after SDS-PAGE in the product estimated to be 5% pure. A N-terminal sequence of the presumed C-proteinase at 95.5 kDa, was finally obtained by concentrating the E-A sample, separating the proteins by a 7% (w/v) polyacrylamide

gel, and blotting to PVDF. However, the N-terminal sequence was presumably blocked by cyclisation of the N-terminal glutamate residue, and so an alternative source of enzyme with higher levels of C-proteinase was sought.

MG63 a human osteosarcoma cell line, which secretes high levels of type I and III collagen, was used as the source for C-proteinase. This cell line which would be relatively easy to culture on a large scale would mean the generation of considerably greater amounts of C-proteinase, whilst eradicating the need for chick embryo dissection; over 1500 doz chicks were dissected in the initial phase of this study. Samples of growth media from MG63 cells had 1.2 fold increased levels of active C-proteinase, compared to chick embryo tendon culture media.

Initially the partial purification of this human C-proteinase, from this novel source, resulted in the purification of only 1130 units of human C-proteinase, a 10 fold decrease on the chick purifications, with major losses of activity in all four chromatography steps. The partially purified C-proteinase was a calcium dependent metalloprotease, which specifically cleaved purified chick and human procollagen at the expected location. As the purifications were taking longer to complete, due to the volume of media, and the length of the electrophoretic assay, the enzyme may have been denatured amounting in an eventual overall loss of activity.

A major loss of activity occured at the concanavalin A-Sepharose step. From the sequence of human BMP-1, 26 potential N-linked glycosylation sites are available in the C-proteinase. With 3.5% of residues having sites for potential N-glycosylation.

The human C-proteinase could be termed highly glycosylated which could account for its high affinity to the concanavalin A-Sepharose column. These losses of activity were not observed during the chick purifications, but were encountered when C-proteinase was purified from mouse fibroblasts (Kessler & Adar, 1989). The enhancer molecule may be required for activity of human C-proteinase, and it may have been separated from the C-proteinase during purification. Further evidence towards the requirement of the enhancer molecule, for activity, is that the enhancer was co-purified with the E-B form of MG63 C-proteinase.

This thesis discusses how difficult it was to purify C-proteinase, firstly from an apparent abundant source using chick tendon, and secondly from MG63 cell growth media. The end result was a partially purified human enzyme which was only 5% pure, from which an unusable N-terminal sequence, which did not match with sequence from human BMP-1 was obtained. This work has to be put into context with all the other research into C-proteinase over the past 20 years. Advances were very slow since Hojima *et al.*, first purified the chick C-proteinase in 1985 (Hojima *et al.*, 1985) and Kessler & Adar purified the mouse C-proteinase along with an enhancer molecule in 1989 (Kessler & Adar, 1989). Not until 1994 was the enhancer molecule identified as a glycoprotein and its structure determined. It was from using this knowledge that the major breakthrough of identifying C-proteinase as BMP-1 was made (Kessler *et al.*, 1996).

A highly sensitive electrophoretic assay was developed, with the addition of 0.02% (w/v) DS, which enabled the detection of C-proteinase in dilute chromatography

fractions and media samples. The DS, when added to the reaction mixture, aggregated the procollagen substrate (Hojima, *et al.*, 1994), enhancing cleavage up to 5 fold. This assay was used to cleave chick and human type I procollagen into pNα(1), pNα(2), C1(1), and C2(1) products. From analysis of the N-terminal sequences of the C1(1) and C2(1) propeptides, the cleavage site of human C-proteinase was the -Ala-Asp- bond, the same as seen with the chick C-proteinase (Dickson *et al.*, 1981). This result showed that the partially purified enzyme was procollagen C-proteinase, and not a non-specific metalloprotease.

Studies on the regulation of C-proteinase synthesis in MG63 cells showed that TGFβ1 upregulated both human C- and N- proteinase. Where an excess of the N-proteinase caused many of the procollagen proα chains to be processed to pCα1/2(I), this was subsequently processed to collagen alpha chains by C-proteinase. However, no increase in collagen synthesis/deposition was observed in TGFβ1 treated cells, possibly because the expression of procollagen mRNAs was not raised to match the levels of the extracellular processing enzymes.

By comparing the CNBr cleavage peptides of both E-A and E-B, the human C-proteinase partially purified from MG63 cells was shown possibly to be the equivalent to the human BMP-1. Recently identified as the same molecule, activities of the two are very different, and the effects of BMP-1 on inducing bone formation may or may not be explained by the C-proteinase activity. BMP-1 is known to be more different from the other BMPs (2-5) which are members of the TGFβ superfamily. One possible explanation is that the bone morphogenetic activity of the original preparation,

containing BMP-1, may have been due to contamination by one of these TGFβ like molecules. This identification opens up an exciting field of research, linking a regulatory molecule firstly, to gene products involved in development, and secondly, to proteases involved in collagen deposition in connective tissue turnover and disease.

The relationship between gene products responsible for cell fate decisions in embryogenesis (Tld), BMP-1 and the TGFβ family of molecules is complex and interesting. BMP-1, co-purified with TGFβ-related proteins, is most like Tld, a gene product essential in embryogenesis for dorsal/ventral patterning in *Drosophila* (Fukagawa *et al.*, 1994), and mutations in Tld are similar to the gene product dpp, which shares 75% homology with BMPs 2 and 4 (TGFβ family members). Also a longer form of Tld, mammalian Tld, is a product of alternatively spliced RNA transcripts of the BMP-1 molecule (Takahara *et al.*, 1994b). Mammalian Tld and BMP-1, which have been detected in most adult tissue, with one notable exception, the brain, are likely to function in development, and possibly in bone formation (Takahara *et al.*, 1994b). C-proteinase which is probably an alternatively spliced RNA transcript of BMP-1, is involved in matrix synthesis in adult tissue. The relationship between the roles for their gene products in embryogenesis and in its formation of bone and soft matrix is yet to be determined.

The work presented in this thesis suggests several areas of further study. As C-proteinase has been identified as BMP-1, the next piece of work would have been in acquiring BMP-1 antibodies, to specifically recognise the enzyme studied here, giving further evidence that human C-proteinase from MG63 cells is related to BMP-1. The

knowledge of the amino acid sequence now allows the expression of recombinant C-proteinase for use in numerous experiments. A number of areas can now be investigated with such a tool. The location, abundance, steps in biosynthesis, regulation by growth factors and control of C-proteinase in normal and fibrotic disorders would be investigated with recombinant C-proteinase.

This recent discovery has come at the end of at least 20 years of research into C-proteinase, where very little progress was made, due to the extreme low levels of expression in tissue. I have identified and partially purified a novel source of human C-proteinase which is upregulated in vitro by TGFβ1, and was shown to be BMP-1-like. It would be fascinating to determine whether this C-proteinase isolated from MG63 cells is identical to BMP-1, or an alternatively spliced form like mTld. With the knowledge of the gene it would be interesting to investigate RNA transcripts in different tissues, and to characterise the activity and function of any different forms of the C-proteinase. Also how the N-glycosylated enhancer is involved in activity and how the E-A and E-B forms are related to each other, and what additional properties or structures give BMP-1 its procollagen processing ability.

APPENDICES

APPENDIX 1: LIST OF SUPPLIERS

Mixture of uniformly labelled ^{14}C-L-amino acids and ^{14}C-formaldehyde from Dupont, research products, Boston, England; DEAE-cellulose pre-swollen microgranular anion exchanger DE52 from Whatman Biosystems, Kent, England; Green A DyeMatrex gel from Amicon, Gloucestershire, England; concanavalin A-Sepharose, heparin-Sepharose CL-6B, sephacryl S-300 from Pharmacia Biotech, Hertfordshire, England; DMEM from ICN Biomedicals Oxfordshire, England; Nutridoma SR from Boehringer Manheim Biochemica, East Sussex, England; FCS, streptomycin/penicillin, PBS, L-glutamine from Gibco BRL, Paisley, Scotland; Sircol collagen assay kit from Biocolor, Belfast, N. Ireland; Recombinant human FGFb, TGFβ1, EGF, IL-4 and IL-1β from R and D Systems, Oxfordshire, England; other standard reagents from Sigma chemical company, Dorset, England, BDH Merck, Dorset, England, and Anachem Bedfordshire, England. PVDF membrane from Applied Biosystems, California, USA and Kodak BIOMAX-MR X-ray film from Eastman Kodak company, New York, USA.

APPENDIX 2

SDS-PAGE

Solutions

Separating gel buffer 1.5 M Tris HCl, pH 8.8

Stacking gel buffer 0.5 M Tris HCl, pH 6.8

Running buffer 384 mM glycine
50 mM Tris
0.1% (w/v) SDS

Sample buffer (1X) 125 mM Tris HCl, pH 6.8
2% (w/v) SDS
0.001% (w/v) bromophenol blue
10% (v/v) glycerol (add after correcting the pH)

Gel solutions

Separating Gel (400 ml)

100 ml of separating gel buffer (pH 8.8)

2 ml of 20% (w/v) SDS

to make 7, 9.5 and 15 % (w/v) polyacrylamide solutions the following was added:

	7%	9.5%	15%
30% acrylamide and 0.8% bisacrylamide (ml)	93	127	200
Distilled water (ml)	203	171	98

For a 16 x 16 cm x 1.5 mm slab gel:

40 ml of separating gel solution

30 µl of TEMED

250 µl of 10% ammonium persulphate

3.5% Stacking Gel (400 ml)

100 ml of stacking gel buffer (pH 6.8)

2 ml of 20% (w/v) SDS

46 ml of 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide

252 ml of distilled water

For a 16 x 16 cm x 1.5 mm slab gel

20 ml of stacking gel solution

30 µl of TEMED

250 µl of 10% (w/v) ammonium persulphate

APPENDIX 3

KREBS BUFFERS

KREBS

NaCl	111.2 mM
KCl	5.4 mM
KH_2PO_4	1.3 mM
$MgCl_2.6H_2O$	1.3 mM
$NaHCO_3$	4.0 mM
Na_2HPO_4	12.5 mM
NaH_2PO_4	3.1 mM
	pH 7.4

KREBS-glucose the above with:

glucose	13 mM

APPENDIX 4

HYDROXYPROLINE ANALYSIS (Woessner, 1961)

Sample Preparation

Samples were hydrolysed in 6M HCl at 110 °C overnight, and evaporated with a gentle flow of nitrogen. The samples were then redissolved in distilled water (or neutralised with NaOH, with the final NaCl concentration in the sample not exceeding 0.4M). A standard curve was constructed with hydroxyproline concentrations from 0 to 20 μg.

Assay

One ml of chloramine T was added to samples, in a final volume of 2 ml, and left at room temperature for 20 min. To each sample, 1 ml of 3.15 M perchloric acid was added. After 5 min at room temperature 1 ml of p-dimethyl aminobenzaldehyde was added to each sample, mixed and incubated at 60 °C for 20 minutes. At a wavelength of 557nm, optical absorbances of each sample was measured using a Pharmacia UltrospecIII UV/visible spectrophotometer. Concentration of hydroxyproline in the samples were then obtained by interpolation using the standard curve. Procollagen concentration in the sample was then determined assuming hydroxyproline constitutes 10.1% of type I procollagen weight.

Solutions

Stock hydroxyproline

10 mg L-hydroxyproline in 100 ml of 0.001M HCl.

Perchloric Acid

3.15 M perchloric acid prepared by diluting 27 ml of 70% $HClO_4$ to a final volume of 100 ml with distilled water.

p-dimethylaminobenzaldehyde (freshly prepared)

A 10% (v/v) solution was prepared shortly before use by adding isopropanol to 2 g of p-dimethylaminobenzaldehyde to give a final volume of 20 ml.

Chloramine T (freshly prepared)

1.41 g of chloramine T in 20 ml distilled water with 30 ml of isopropanol and 50 ml of hydroxyproline buffer.

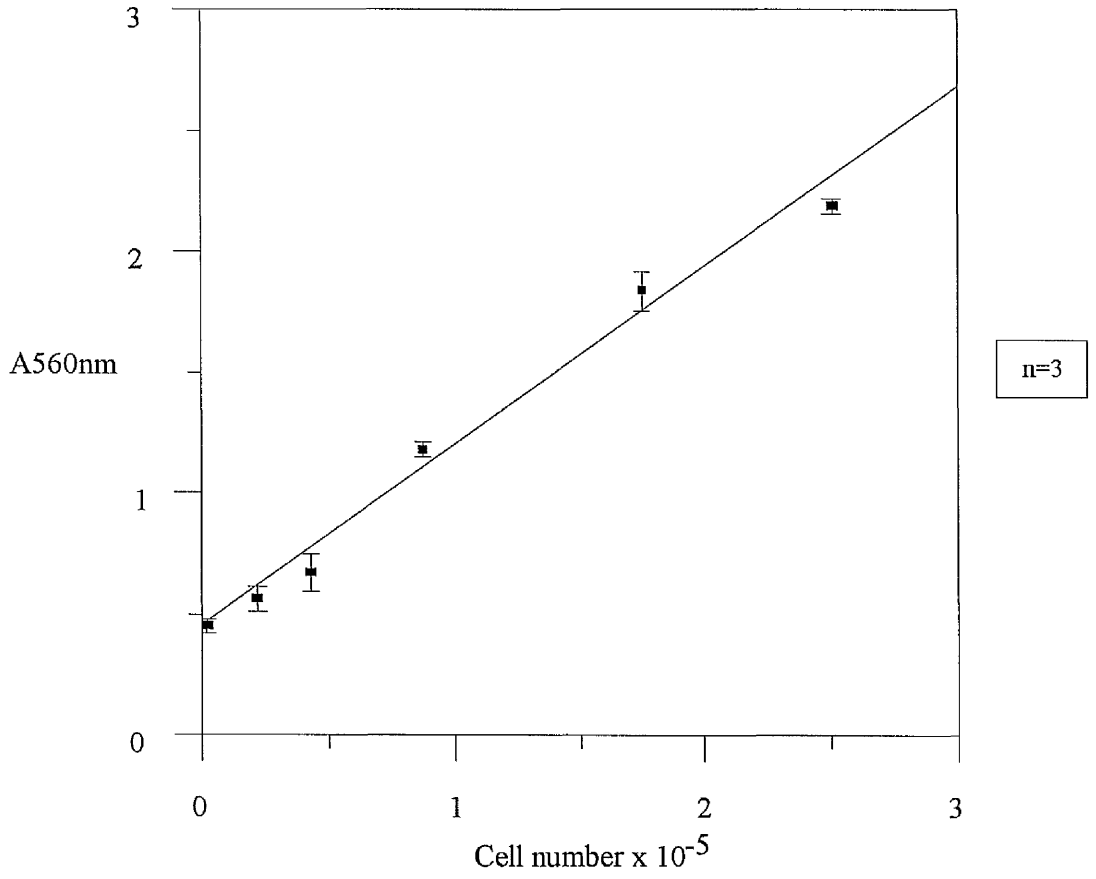
Hydroxyproline buffer	Citric acid monohydrate	5 g
	Glacial acetic acid	1.2 ml
	Sodium acetate trihydrate	120 ml

pH was adjusted to 6.0 at room temperature using either concentrated NaOH solution or 3.4 g NaOH). After adjusting pH final volume brought to 100 ml with distilled water.

APPENDIX 5

MTT ASSAY STANDARD CURVE

Figure A1: Standard Curve of MTT Assay

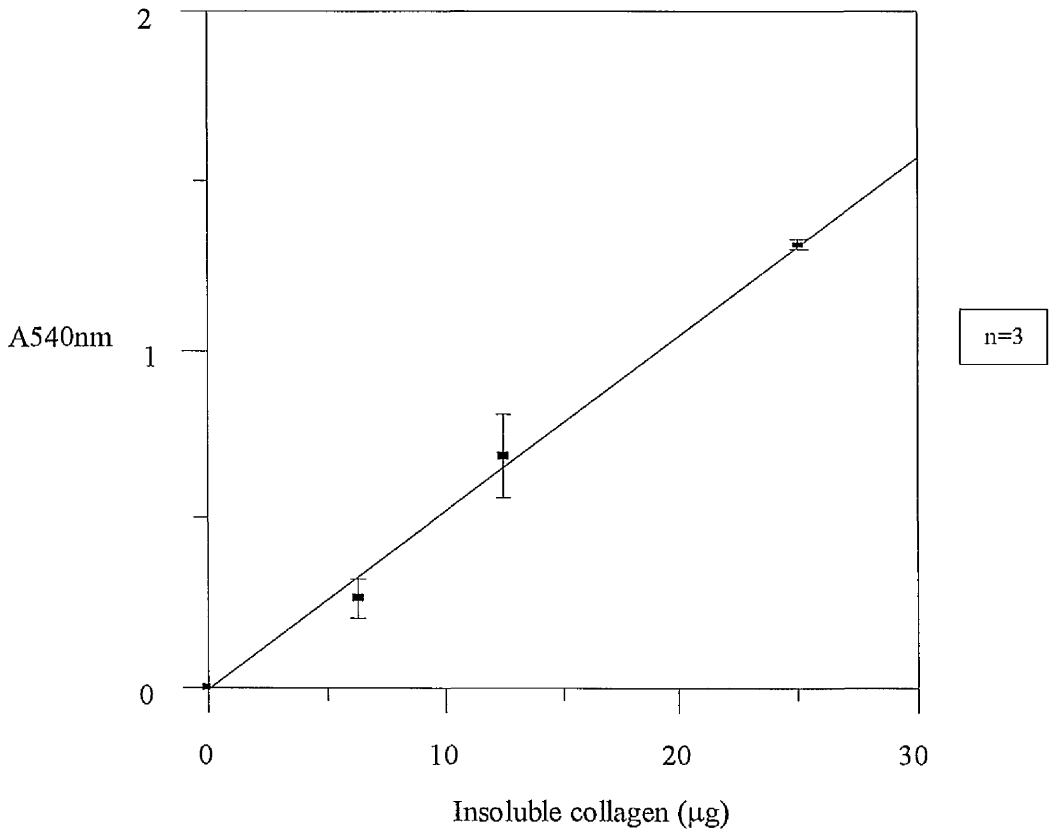


Cell number was determined by interpolating absorbances at 560nm from test samples, incubated with 5 mg/ml MTT solutions for 4 hr., and then solubilised with isopropanol.

APPENDIX 6

SIRCOL COLLAGEN ASSAY STANDARD CURVE

Figure A2: Standard Curve of Sircol Assay



Insoluble collagen (μg) in cell layer was determined by interpolating absorbances at 540nm from test samples, incubated 0.5 ml 0.1% (w/v) Sirius red in saturated piric acid, for 1 hr, and then solubilised with 0.5 ml of 0.5 M NaOH.

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