Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 329-337

STABILISATION OF PURIFIED HUMAN COLLAGENASE BY SITE-DIRECTED MUTAGENESIS

Mary C. O' Hare,*Valerie A. Curry, Rebecca E. Mitchell, and Tim E. Cawston

Rheumatology Research Unit, Addenbrookes Hospital, Cambridge CB2 2QQ, United Kingdom

Received September 26, 1995

During purification, human fibroblast collagenase breaks down into two major forms, an N-terminal 22000/25000-M_r fragment and a C-terminal 27000-M_r fragment; the most likely mechanism being autolysis. The cleavage site has been identified (Pro269- Ile270) and in an attempt to obtain full-length human collagenase (i.e.,M_r 42570), this cleavage site and another potential cleavage site (Ala258- Ile259) have been mutated by PCR- directed mutagenesis: Ile270Ser and Ile259Leu. The mutated cDNA was then cloned into the expression vector, pGEX2T, and expressed in Escherichia coli as a fusion protein with glutathione-S-transferase (GST). After cleavage with factor Xa, the mutated collagenase was purified on a peptide hydroxamic acid affinity column. The mutated recombinant collagenase is stable, remains full length and retains the ability to cleave collagen.

Collagenase (matrix metalloproteinase 1, MMP1) is a neutral proteinase able to initiate breakdown of the interstitial collagens types I, II and III [1,2]. Collagenase plays a key role in the constant remodelling of collagen that occurs in both normal and diseased conditions. Thus, mechanisms concerning the regulation of collagenase at both the protein and the gene level are of prime importance to our understanding of connective tissue metabolism.

Collagenase is synthesised as a preproenzyme, including a 19 amino acid residue signal peptide. The major secreted protein is a proenzyme of M_r 51929, which can be N-glycosylated (via Asn120 and Asn143) to a minor form of M_r approximately 57000. Procollagenase can be activated, at least in vitro, by proteinases (e.g.trypsin or plasmin) and mercurials (e.g. 4-aminophenylmercuric acetate). Upon activation, the propeptide is cleaved [3]. The active enzyme has M_r 42570 and contains both glycosylation sites and 2 cysteine residues. Once activated, human collagenase breaks down into two major forms, an N-terminal 22000/25000-M_r fragment and a C-terminal 27000-M_r fragment [4,5]. The 25000-M_r fragment has been

*Correspondence to: Dr. M.C. O' Hare, Rheumatology Research Unit, Addenbrookes Hospital, Cambridge CB2 2QQ, UK. Fax (01223)217838.

Abbreviations: oligo, oligodeoxyribonucleotide; PMSF, phenylmethylsulphonylfluoride; PolK, Klenow.

0006-291X/95 $12.00

shown to be a glycosylated form of the 22000-M_r fragment [5]. The N-terminal fragment retains the active site, but loses its collagen specificity [5,6], whereas the C-terminal fragment has no enzyme activity but retains the ability to bind to collagen [6,7].

To try and stabilise the full length collagenase, the cleavage sites identified in our laboratory were mutated by PCR-directed mutagenesis and the mutated cDNA was then expressed in E.coli as a fusion protein with glutathione-S-transferase (GST). In this paper we describe the mutagenesis, expression in E.coli, and the purification of the mutated human collagenase which when purified remains full length and retains the ability to cleave collagen.

MATERIALS AND METHODS

Materials

The expression vector pGEX2T was obtained from Pharmacia, Milton Keynes, Bucks., U.K.; restriction endonucleases, T4 DNA Ligase and Klenow from Boehringer Mannheim, Lewes, East Sussex, U.K.; PMSF and lysozyme from Sigma, Poole, Dorset, U.K. Prolylleucylglycinehydroxamic acid was synthesised by Bachem, Germany. All other chemicals were analytical grade reagents from Fisons, Loughborough, Leics., U.K.

Methods

Construction of pGEX2T/ collagenase expression vector.

For expression in E.coli, the expression vector pGEX2T was used. This expression system [8] allows the expression of foreign genes as fusion proteins with glutathione-S-transferase (GST). Expression is under the control of a *tac* promoter which enables inducible, high level production of fusion proteins. To enable the mature active form of collagenase to be produced, the pGEX2T vector was modified by the addition of a synthetic double stranded oligomer as a *Bam*HI-*Eco*RI fragment (Fig.1). This 20-mer encodes a factor Xa cleavage site and a *Nru*I restriction endonuclease site, which creates blunt ends after cleavage, thus enabling a precise fusion directly in front of the collagenase gene. The conversion of Ile259 -> Leu and Ile270 ->Ser (bonds numbered from the N-terminal methionine residue) was achieved in two steps by using the template cDNA (isolated as described in [9] and the polymerase chain reaction [10]. The cDNA was subjected to 30 rounds of amplification with Taq polymerase in the presence of oligonucleotides 3 and 4 (Fig.2A&B) to generate a 486bp N-terminal half of the collagenase cDNA with a 5' TTT coding for the N-terminal phenylalanine of mature collagenase, and a 3' *Bgl*II restriction site. In parallel, the cDNA was subjected to 30 rounds of amplification with Taq polymerase in the presence of oligonucleotides 5 and 6 (Fig.2A&B) to generate a 633bp C-terminal half of the collagenase cDNA with a 5' *Bgl*II restriction site and a stop codon at the 3' end. The amplified products were separated from unincorporated nucleotides and oligos on a primerase column, modified by PolK, [11] and digested with *Bgl*II. The modified pGEX2T vector was digested with *Nru*I and ligated with the 486bp and 633bp fragments of collagenase in a 3-way ligation. The mutated sequence was verified by double stranded dideoxy methodology (Fig. 2C).

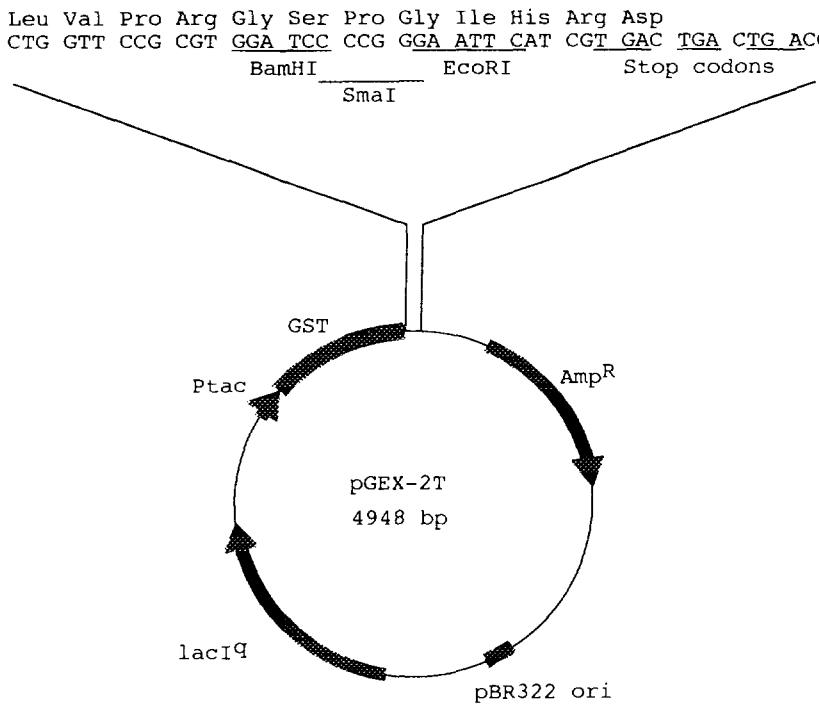
Synthesis of GST-Collagenase fusion protein in E.coli.

Recombinant plasmids expressing GST-Collagenase fusion protein from pGEX2T/Collagenase were introduced into E.coli strain XL1-Blue [12]. Cells were harvested, resuspended in Tris-$CaCl_2$ buffer (0.1MTris-HCl pH7.9/10mM $CaCl_2$) containing PMSF and lysozyme, lysed by sonication and the lysates cleared by centrifugation at 34000 x g for 45 min. at 4°C. Supernatant and pellet fractions were analysed by 0.1% SDS-10% PAGE and stained with Coomassie Brilliant Blue G-250. A polypeptide of molecular weight approximately 68kDa was detected in the pellet fraction only (Fig.3 lane 2). This polypeptide was not detected in cells transformed with vector pGEX2T alone (Fig.3 lane1).

Extraction of the GST-Collagenase fusion protein from the cell pellet.

The cell pellet was extracted by suspension in urea buffer (8M urea/0.5M Tris-HCl pH7.9/0.5M NaCl/10mM $CaCl_2$/30mM -mercaptoethanol/1mM PMSF). The suspension was vortexed vigorously and dialysed against 50mM Tris-HCl pH7.9/0.5M NaCl at 4°C for 48 hr

A



B

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                    Ser Ile Glu Gly Arg Glu
5' Oligo   5'- GA TCC ATC GAG GGT CGC GAG  - 3'
                                  Nru I

3' Oligo   5'- A ATT CTC GCG ACC CTC GAT G - 3'
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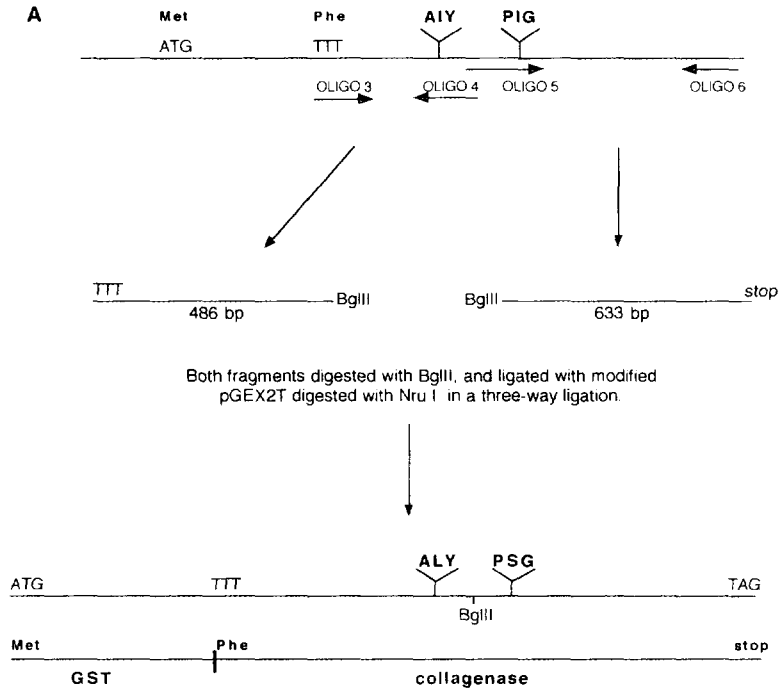
Fig. 1. General features of the pGEX2T vector and sequence of synthetic double stranded oligomer.
A. Map of fusion vector pGEX2T: Ptac, *tac* promoter; GST, glutathione-S-transferase gene.
B. The oligos were annealed together and ligated into the *Bam*HI-*Eco*RI cut pGEX2T vector to create a factor Xa cleavage site and a unique *Nru*I restriction site.

with 6 buffer changes. The extract was centrifuged at 13000g for 20 min. at 4°C. The supernatant, S_2, was then enriched for the GST-collagenase fusion protein.

Purification of Mutated Human Collagenase on PHA-Sepharose.
Mutated collagenase was purified from E.coli (large scale fermentation was kindly performed by Helen Edwards, SmithKline Beecham Pharmaceuticals, Great Burgh, Surrey, U.K.). Prolylleucylglycinehydroxamic acid (PHA), as described in [13] was coupled to activated CH-Sepharose 4B according to the recommended Pharmacia procedure. The column was equilibrated in 0.1M Tris-HCl pH 7.5/10mM $CaCl_2$/0.5M NaCl/0.02% NaN_3. The urea-extracted supernatant containing the GST-collagenase fusion protein was incubated with factor Xa at 4°C and dialysed for 48h against 50mM Tris-HCl pH 8.0/0.1M NaCl/1mM $CaCl_2$. When the cleavage was complete, i.e. no longer any fusion protein remaining, this fraction was then dialysed against PHA column buffer and applied to the PHA column (2.6 x 9.5cm). The column was washed until the $A_{280} = 0$, then the mutated collagenase was eluted with 50mM Tris-HCl pH 9.0/0.5M NaCl/10mM $CaCl_2$. Fractions were collected and assayed for protein



B

Oligonucleotide Sequences

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                    Phe Val Leu Thr Glu Gly Asn
Oligo 3.      5' -C TTT GTC CTC ACT GAG GGA AAC CC- 3'

                  Gln Ser Arg Gly Tyr Leu Ala Gln Ile
Oligo 4.      5'-TTG AGA TCT TCC ATA TAG GGC TTG GAT- 3'
                    BglII

                     Arg Ser Gln Asn Pro Val Gln Pro Ser
Oligo 5.      5'-GA AGA TCT CAA AAT CCT GTC CAG CCC AGC G- 3'.
                    BglII

                  Thr Ser     Asn Lys Arg Cys Asn Phe
Oligo 6.      5'-GGT CGA CTA ATT TTT CCT GCA GTT GAA C- 3'.
                      stop
```

C

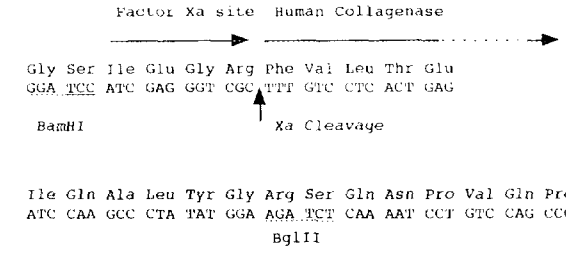

Fig.2. Flow diagram of Mutagenesis.
A. Collagenase cDNA was modified by PCR in two steps: Oligos 3 &4 were used to create a 486bp N-terminal half of collagenase with N-terminal phenylalanine, Leu259 (instead of Ile259) and a 3' *Bgl*II restriction site. Oligos 5&6 were used to create a 633bp C-terminal half with a 5' *Bgl*II restriction site, Ser270 (instead of Ile270) and a stop codon. The fragments were ligated into pGEX2T in a 3-way ligation. The amino acids are numbered from the N-terminal methionine residue.
B. Sequence of oligonucleotides 3,4,5 & 6.
C. The ligated collagenase fragments were sequenced to confirm the mutations were correct. This figure shows the sequence at the junction of the factor Xa cleavage site and the N-terminus of collagenase and at the BglII junction.

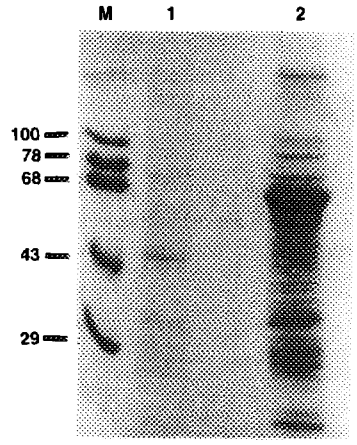

Fig.3. Expression of GST-Collagenase fusion protein in E.coli.
E.coli pellets were taken up in SDS sample buffer, analysed by 0.1% SDS-10% PAGE and proteins stained with Coomassie Brilliant Blue. Lanes: 1, pellet from uninduced E.coli cells transformed with pGEX2T alone; Lane 2, pellet from E.coli cells transformed with pGEX2T/collagenase. M, molecular weight markers (in kDa).

and for collagenase activity using the collagen fibril assay. Fractions containing collagenase activity were pooled and analysed by SDS-PAGE (Fig 4). Table I shows the purification scheme. The purified protein was N-terminal sequenced to confirm the F-V-L-T sequence for the native active enzyme (M. O'Hare, unpublished results).

Purification of Wild Type Human Collagenase.
Wild type human collagenase was purified from the culture medium of WI38 foetal lung fibroblasts (kindly provided by SmithKline Beecham). Purification was achieved by using Zn^{2+}-chelate chromatography as described in [5], followed by peptide hydroxamic acid affinity chromatography as described above. The wild type collagenase was eluted with 50mM Tris-HCl pH 9.0/0.5M NaCl/10mM $CaCl_2$ and fractions containing collagenase activity were pooled and analysed by SDS-PAGE (Fig.4 lane 2).

Table I

Purification of recombinant human collagenase from *E. coli* [1]

	Collagenase Activity (Units)	Protein (mg)	Specific Activity (Units/mg)	Yield (%)	Purification (fold)
Urea extracted supernatant cleaved with factor Xa[2]	41093	3293	12.5	100	1
PHA-Sepharose	22470	7.7	2918	55	233

[1]From a 10 litre fermentation.
[2]No collagenase activity detected before this step in the purification procedure.

Collagen Fibril Assay
The assay was performed as described in [14]. One unit of enzyme activity digests 1μg of substrate/min. at 37°C.

Specific cleavage of Collagen
Calf-skin type I collagen (1mg/ml; $1x10^8$ cpm/mg) was iodinated as described in [15]. This material (1μg)was incubated with mutated human collagenase (0.25μg), wild type human collagenase (0.25μg) and with factor Xa (1 unit). The reaction was carried out at 25°C for 18h in the presence of 0.1M Tris-HCl pH7.5/0.2MNaCl/5mM $CaCl_2$/1M glucose. The reaction products were then analysed on an 0.1% SDS-8% polyacrylamide gel and subjected to autoradiography (Fig. 5).

RESULTS

Purification of the recombinant mutated human collagenase

Purified wild type human fibroblast collagenase breaks down, with cleavage of the Pro269-Ile270 bond and possibly the Ala258-Ile259 bond. Wild type porcine synovial collagenase also undergoes autolysis, but at the Ala258-Ile259 bond only [16]. In an attempt to obtain full length human collagenase, the Pro269-Ile270 bond in the human cDNA was mutated to Pro269-Ser270 (the equivalent in the porcine cDNA, [17] and the Ala258-Ile259 bond was mutated to Ala258-Leu259 (the equivalent in the rat sequence, [18]. (See Fig.2). This mutated human collagenase cDNA was then cloned into the expression vector pGEX2T and expressed in E.coli as a fusion protein with GST. This expression system has been used successfully for the expression and purification of more than 30 different eukaryotic polypeptides. In the majority of cases, the fusion proteins were expressed as soluble proteins and could be purified from crude bacterial lysates under non-denaturing conditions by affinity chromatography on immobilised glutathione. However, in this case the GST-collagenase fusion protein was expressed in an insoluble form and it was necessary to solubilise the fusion protein by urea extraction (See Methods). During refolding, the fusion protein was cleaved between the GST and the collagenase (M. O' Hare unpublished data). This ruled out the possibility of purification on Glutathione-Sepharose, so the collagenase was incubated with factor Xa and then purified on a PHA-Sepharose column (see Methods). The mutated collagenase bound to and was eluted from this matrix as efficiently as the wild type human collagenase. When run on a 0.1%SDS-12.5% polyacrylamide gel the mutated collagenase was pure, had an apparent molecular weight of 42kDa (i.e. full length) and no autolysis products could be seen (Fig.4). The specific activity of the mutant collagenase is 2000U/mg, which is similar to that of wild type human collagenase (I.M.Clark unpublished data).

Purification of wild type human collagenase

Wild type human collagenase was purified from WI38 foetal lung fibroblasts at the same time as the recombinant human collagenase. As can be seen from Fig.4 lane 2 ,the majority of the collagenase has undergone autolysis to yield the N-terminal M_r22000/25000 fragment. The C-terminal 27000-M_r-fragment did not bind to the PHA-Sepharose as it no longer contains the active site.

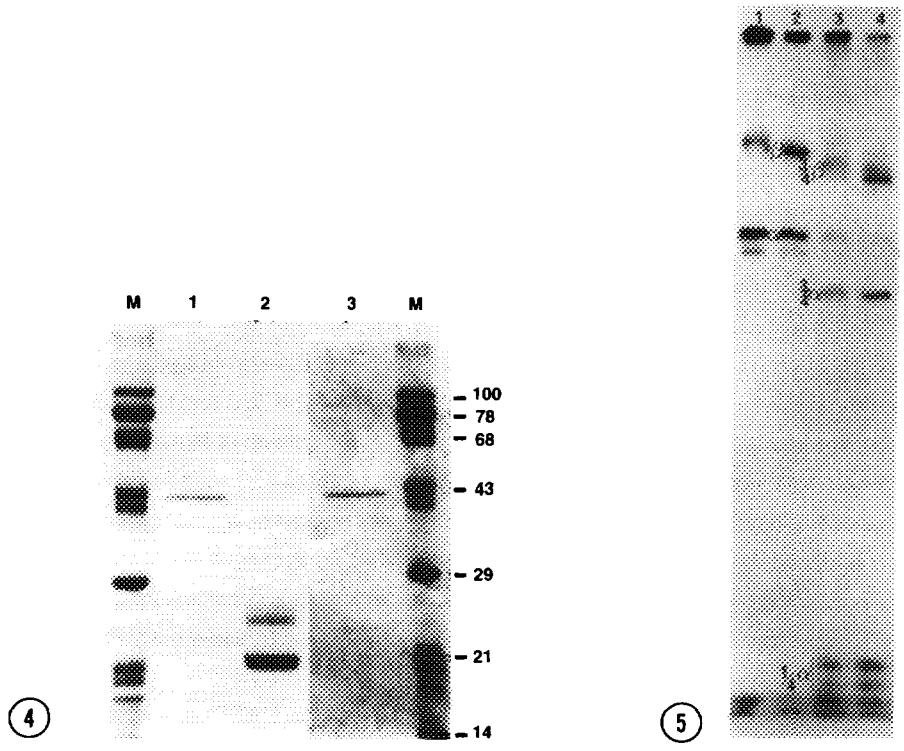

Fig.4. 0.1%SDS/12.5% polyacrylamide gel electrophoresis of purified mutated and wild type human collagenase.
Lane 1, Purified mutated human collagenase eluted from PHA-Sepharose at pH 9.0; Lane 2, Purified wild type human collagenase eluted from PHA-Sepharose at pH 9.0; Lane 3, Purified mutated human collagenase after storage for 3 months at 4°C; M, Molecular weight markers (in kDa).

Fig.5. Specific cleavage of collagen.
^{125}I-labelled collagen (1µg), prepared as described (Bolton and Hunter, 1973) was incubated with recombinant mutated human collagenase (0.25µg), wild type human collagenase (0.25µg) and factor Xa (1 unit) in 0.1M Tris.HCl pH7.5/0.2M NaCl/5mM CaCl2/1M glucose at 25°C for 8hr. After 0.1% SDS -8% PAGE under reducing conditions, followed by autoradiography, the characteristic 3/4 and 1/4 fragments produced from the α and β chains of collagen could be seen. Lane 1; calf-skin type I ^{125}I-labelled collagen. Lane 2; ^{125}I-labelled collagen and factor Xa. Lane 3; ^{125}I-labelled collagen and wild type human collagenase. Lane 4; ^{125}I-labelled collagen and mutated human collagenase.

Specific cleavage of collagen

Cleavage of type I collagen is a very specific assay for collagenase as only full length collagenase can cleave type I collagen into 3/4 - 1/4 fragments at the characteristic collagenase sensitive site (the N-terminal 22000/25000-Mr fragment can not cleave collagen). As shown in Fig.5 the mutant collagenase yields the expected 3/4 and 1/4 collagen fragments (Fig.5 lane 4). The wild type human collagenase also cleaved collagen into 3/4 -1/4 fragments (Fig.5 lane 3). Although the preparation contained predominantly autolysis products, sufficient full length enzyme was still present to cleave collagen. When the wild type human collagenase was concentrated 50 fold and run on an SDS gel, some full length collagenase was just detectable (results not shown).

DISCUSSION

The N-terminal and C-terminal collagenase fragments are tightly folded domains held together by a linker peptide (residues 248-278), which is thought to be of poor secondary structure. This linkage, which is a feature of all the MMPs except MMP7, appears an obvious target for proteolysis and autolysis and if it is cleaved the N-terminal catalytic domain becomes a non-specific proteinase [4,5,6], similar to matrilysin, MMP7 [19,20]. Collagen can no longer be cleaved but the N-terminal domain retains its ability to cleave casein and gelatin and gains the ability to superactivate procollagenase. Stromelysin, MMP3, also undergoes autolysis in this hinge region [21,22,23], as does neutrophil collagenase, MMP8, [24].

Several groups have tried unsuccessfully to obtain full length human collagenase, which breaks down slowly even in the presence of protease inhibitors (unpublished data). Recently, two independent structural analyses of the N-terminal "catalytic" domain of human fibroblast collagenase have been published [25,26]. This domain of about 150 amino acids represents less than half of the full length collagenase molecule, has no activity against collagen and is weakly inhibited by TIMP. The stuctures obtained show that the active site is too small to accommodate all 3 collagen chains; it is possible that one collagen chain at a time might be cleaved, but a structure for full length collagenase is needed to ascertain the active site conformation.

TIMP binds tightly to the active collagenase molecule, whereas TIMP binds weakly to the N-terminal collagenase domain [6,7]. This suggests that the interaction with TIMP involves both the N-terminal and C-terminal domains of collagenase. This recombinant mutated full length human collagenase can now be used to investigate e.g. the interaction of TIMP binding to collagenase, structural studies of the collagenase:TIMP-1 complex, substrate binding and cleavage.

In conclusion, a cDNA encoding human synovial cell collagenase was mutated in an attempt to stabilise the collagenase and stop autolysis. The mutated collagenase was then expressed in E.coli, and purified in a single step on a peptide hydroxamic acid affinity column. The purified mutated collagenase is active, stable and has remained uncleaved, with no signs of autolysis for more than 3 months at 4°C (Fig. 4). We conclude that mutagenesis of the cleavage sites within the linker peptide has stopped autolysis and we now have an expression system which produces the large amounts of active full length human collagenase required for our biochemical and biophysical studies.

ACKNOWLEDGMENTS

We thank the Arthritis and Rheumatism council for funding, SmithKline Beecham for large scale E.coli fermentation, Dr. Connie Brinckerhoff for the human collagenase cDNA clone; Dr. I.M. Clark for critical review of the manuscript and Miss Rebecca Harrall for technical assistance. Miss Rebecca Mitchell was funded by a Wellcome Vacation Scholarship.

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