PRODUCTION OF HUMAN PROTEIN TIMP-2: A HIGHLY EFFECTIVE ANTI-AGING INGREDIENT

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Summary

The matrix metalloproteinases (MMPs) are a family of enzymes responsible for degrading connective tissue. MMPs catalyze the breakdown of collagen from the extracellular matrix, leading to wrinkle formation and accelerated skin aging. Furthermore, ultraviolet irradiation causes increased expression of certain MMPs. In the extracellular matrix turnover, MMPs are interacting with endogenous regulators named tissue inhibitors of metalloproteinases (TIMPs). Using peptide substrate assays, it has been demonstrated that TIMP-MMP complexes interact highly specifically with K_i values of 10^{-9}-10^{-16} M. Therefore applications for TIMP as inhibitor of collagen degradation are suggested for cosmetic anti-aging products to prevent wrinkle formation and loss of elasticity. To date four TIMP proteins (TIMP-1, TIMP-2, TIMP-3 and TIMP-4) have been identified which show a high degree in sequence similarity.

The production of human TIMP-2, a 194-residue nonglycosylated protein, was performed by fed-batch culture of *Escherichia coli*. TIMP-2 accumulated in the bacterial cells in an insoluble form as inclusion bodies. The inclusion bodies were solubilized and the protein refolded to yield the native TIMP-2 in the active form. The integrity of the protein was confirmed by mass analysis, Edman sequencing and gel shift experiments with authentic samples. The inhibitory activity of the refolded and purified TIMP-2 was demonstrated with MMP-1 and MMP-2 assays using synthetic fluorogenic peptide substrates.

Introduction

In normal conditions matrix metalloproteases (MMP) are involved in controlled tissue remodelling and responsible for the degradation of collagen as part of the extracellular matrix, in particular collagen type I, which is the major structural protein in skin. However, it is also known that in skin aging the MMP activity is increased whereas the collagen synthesis is reduced [1]. This situation results in a depletion of collagen in aged skin and it is thought to be a major factor that underlies the alterations observed for aging e.g. skin sagging or the formation of wrinkles.

In addition, the exposure to sunlight (UV irradiation) leads to an immediate and strong increase in MMP activity whereas the concentration of TIMP, the natural inhibitor of MMPs, remains unchanged

or is reduced [2]. Hence, the repetitive UV stimulation results in an even more accelerated skin damage called photodamage (photoaging) which is also relevant for young skin [3]. Besides UV irradiation it was also demonstrated that cigarette smoking increases the MMP activity in skin [4]. Recently it has been found that collagen, fragmented by MMP may downregulate the neosynthesis of collagen and thus impair the situation of damaged skin [5].

The tissue inhibitor of matrix metalloprotease-2 (TIMP-2) is one of the natural endogenous inhibitors specific for all the MMPs that are relevant in skin aging, in particular for MMP-1, -2 and -3. Here, we demonstrate the biotechnological production (bacterial expression) of the human TIMP-2 protein, the processing to high purity and the integrity of the isolated protein compared to natural TIMP-2. Furthermore we show that our recombinant hTIMP-2 is a potent inhibitor of MMP-1 and MMP-2 in the lower nanomolar range.

We suggest TIMP-2 to use as anti-aging ingredient for skin to inhibit intrinsic and extrinsic aging. In other words TIMP-2 could serve as ingredient for a long term treatment to correct the imbalance between collagen neosynthesis, MMP activity and inhibition. Also, TIMP-2 may be applicable as protection factor in the acute phase induction of MMP caused after UV irradiation of the skin.

Materials and Methods

Expression of TIMP-2

Escherichia coli B-type strain harboring a pBR322 derived vector with the human timp-2 gene was cultured in fed-batch procedure in a 50 l pilot fermenter (Mavag, Switzerland). Initially, a seed culture was obtained from an overnight culture in LB medium (2 l) at 37°C in shaker flasks. The seed culture was inoculated into a modified M9 medium (20 l) with additional Plant Peptone E1 (10 g/l, Organotechnie SA, France), glucose (4 g/l), ampicillin (50 μg/l). The fermentation was run at 30°C in batch mode until the glucose of the initial culture was depleted. Subsequently, the feeding solution (24% glucose, 0.5 g/l $Mg_2SO_4 \cdot 7H_2O$) was added at a constant feed rate which was increased by stepwise changes from 1 ml/min up to 4 ml/min. The glucose content in the culture medium was analyzed on-line using the YSI 2700 Glucose Analyzer with a continuous sampling removal system (Biopem, B.Braun Biotech GmbH, Germany).

Downstream processing

The cells were harvested by centrifugation yielding 580 g wet cell weight and were stored at -80°C until needed for processing. An aliquot of cell pellet (300 g) was disrupted by ultrasonication (Telsonic Ultrasonics device DG-100). TIMP-2 protein was isolated from inclusion bodies and refolded according to [6]. After diafiltration the TIMP-2 concentrate was purified on a preparative Mono-Q-HR_10/10 column (Amersham Biosciences Ltd, UK) by anion exchange chromatography resulting in 480 mg active protein.

Analytical SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed using commercially available 4-20% Tris-glycine gels (Invitrogen, EC6025) stained in Coomassie brilliant blue. The probes were transferred in Tris-glycine SDS sample buffer (Invitrogen LC 2676) with additional 3% R,R-dithiothreitol (DTT) for reducing conditions.

Edman degradation

An isolated TIMP sample (100 pmol) was run on a SDS polyacrylamide gel and electroblotted on a PVDF membrane. The proteins were stained with a 0.1% solution of Coomassie brilliant blue S-250 and destained with an aqueous solution of 50% methanol. The purified TIMP-2 band was excised from the dried membrane and analysed on an ABI 476A Protein Sequencer using standard techniques.

MALDI-TOF-MS analysis

The test sample (10 pmol) was analyzed together with sinapinic acid as matrix substance and insulin as internal standard on a Voyager-DE- Elite device in the positive ion mode. The number of laser shots was 50 per spectrum.

MMP-Inhibition assay

Inhibitor activities of recombinant TIMP-2 were assayed against human MMP-1 and MMP-2, respectively. The proenzyme MMP-1 (0.31 μM, Calbiochem 444208) or MMP-2 (0.16 μM, Calbiochem 444213) were activated by 4-aminophenylmercuric acetate (0.34 mM, Sigma A9563) in reaction buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 20 μM $ZnSO_4$, 0.05 % Brij 35) for 3 h at 37°C. The reaction mixture was diluted with an appropriate amount of reaction buffer and transferred into a 96-well plate. The reaction was started with the addition of the fluorogenic substrate (10 μM RAE(pNA)LGLPKAbz, Pentapharm Ltd, Switzerland) to the activated enzyme solution (10 nM MMP-1; 5 nM MMP-2 final concentration) and the TIMP-2 inhibitor solution ranging from 5 to 20 nM final concentration. Time course analysis was performed at 37°C for 1 h measuring the fluorescence at 425 nm with an excitation wavelength of 340 nm (Tecan SPECTRAFluor Plus).

Results and Discussion

The recombinant human TIMP-2 gene isolated from cDNA of melanoma cells was expressed in

E.coli using a pBR322 derived vector. The fermentation of the *E.coli* culture was carried out in a fed-batch procedure in minimal medium and yielded ~25 g wet cell weight per liter fermentation volume (Figure 1). The SDS-PAGE analysis showed an increase of TIMP expression during the fermentation course. An amount of 5% of the total protein content was estimated for the TIMP protein (Figure 2). The construct formed insoluble inclusion bodies which were isolated, subsequently processed utilizing the refolding method by *Wiliamson* [6] and purified using simple anion exchange chromatography under native conditions. The native TIMP-2 protein contains six intramolecular disulfide bridges. The chromatographic purification resulted in two fractions, Peak 1 (Figure 3, sharp peak) and Peak 2 (Figure 3, broad peak) which both corresponded to TIMP-2 protein when analyzed by reducing SDS-PAGE (Figure 4A). However, the migration pattern of the two fractions was distinctly different in non-reducing conditions (Figure 4B). In contrast to the reduced TIMP-2 protein which migrated to an apparent mass of almost 30 kDa, the apparent mass for the non-reduced form was about 22 kDa, indicating more compact protein structures due to the disulfide cross-linking. More importantly, in the fraction Peak 2 a distinct smear band of higher molecular weight appeared besides the 22 kDa band. Therefore we conclude that the fraction Peak 2 contains considerable amounts of TIMP-2 protein aggregates randomly cross-linked by intermolecular disulfide bridges during the process of refolding. Further evidence was given by the strongly reduced MMP1 and MMP-2 inhibition activity of fraction Peak 2 relative to fraction Peak 1 (results not shown).

From one liter of cell culture 34 mg of pure (>95%) and active recombinant TIMP-2 (Peak 1) was recovered. The overall recovery of folded protein was between 20-30%.

Amino-terminal sequencing of the protein from Peak 1 revealed a single amino terminus with the predicted sequence, CSCSPVHPQQ (mature TIMP-2 [Homo sapiens], NCBI Acc. No. NP_003246). The molecular mass of the protein was confirmed by MALDI-TOF-MS analysis (Figure 3, insert). The Edman sequence, the molecular mass and the gel shift mobility of the recombinant TIMP-2 protein (Peak 1) clearly demonstrated the correct chemical integrity as anticipated from literature.

In addition to the structural analysis we performed preliminary inhibition studies of MMP-1 (Figure 5, 10 nM) and of MMP-2 (Figure 6, 5 nM) with our TIMP-2 and a fluorogenic peptide substrate. The positive control consisted in the fully active enzyme-substrate solution without any inhibitor. Both enzymes showed complete inhibition, when 23 nM of Peak 1 protein was used. At a concentration of 10 nM TIMP-2, both, the MMP-1 and MMP-2 activities were reduced by approximately 30%. Thus, a more effective inhibition of MMP-1 was observed compared to MMP-2, since the double amount of enzyme concentration was applied for MMP-1.

Conclusions

In this paper we showed the successful production of the recombinant human TIMP-2 protein in *E. coli* resulting in the active form of the inhibitor which was confirmed by the MMP assays. Due to the

versatile specificity of TIMP-2 on the most important MMPs (MMP-1, MMP-2, MMP-3) for aging, the application of TIMP-2 as cosmeceutical is of great interest. In order to test the in vivo efficacy of TIMP-2, it is important to evaluate the potential of its bioavailability to skin cells with appropriate formulations.

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References

[1] Varani, J., Warner, R.L., Gharaee-Kermani, M., Phan, S.H., Kang, S., Chung, J.H., Wang, Z.Q., Datta, S.C., Fisher, G.J., Voorhees, J.J., Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin, *J. Invest. Dermatol.* **114** (2000), 480-486

[2] Kawaguchi, Y., Tanaka, H., Okada, T., Konishi, H., Takahashi, M., Ito, M., Asai, J., The effects of ultraviolet A and reactive oxygen species on the mRNA expression of 72-kDa type IV collagenase and its tissue inhibitor in cultured human dermal fibroblasts, *Arch. Dermatol. Res.*, **288** (1996), 39-44

[3] Fisher, G.J., Wang, Z.Q., Datta, S.C., Varani, J., Kang, S., Voorhees, J.J., Pathophysiology of premature skin aging induced by ultraviolet light, *N. Engl. J. Med.*, **337** (1997), 1419-1428

[4] Lahmann, C., Bergemann, J., Harrison, G., Young, A.R., Matrix metalloproteinase-1 and skin aging in smokers, *The Lancet,* **357** (2001), 935-936.

[5] Fligiel, S.E., Varani, J., Datta, S.C., Kang, S., Fisher, G.J., Voorhees, J.J., Collagen degradation in aged/photodamaged skin in vivo and after exposure to matrix metalloproteinase-1 in vitro, *J. Invest. Dermatol.,* **120** (2003) 842-8

[6] Wiliamson, R.A., in *Methods in Molecular Biology*, Matrix Metalloproteinase Protocols, Clark I., Humana Press Inc., Totowa, USA, Vol 151 (2001), p. 257-265

FIGURE 1

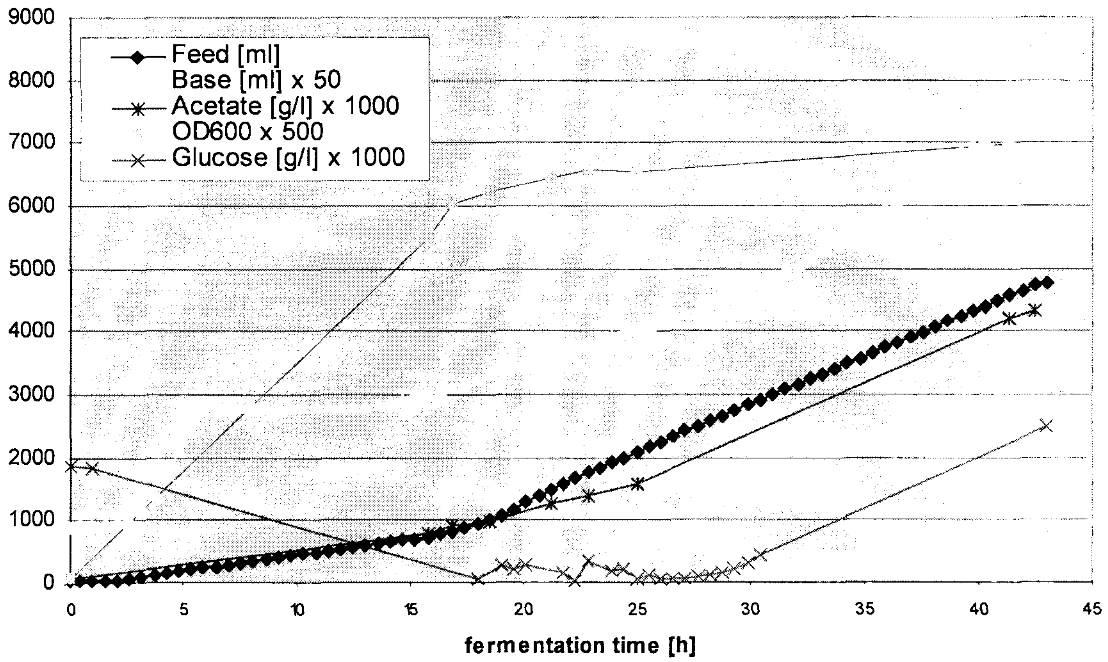

FIGURE 1: Time profiles of feeding rate, base addition, optical densities (OD) at 600 nm, acetate and glucose concentration.

FIGURE 2

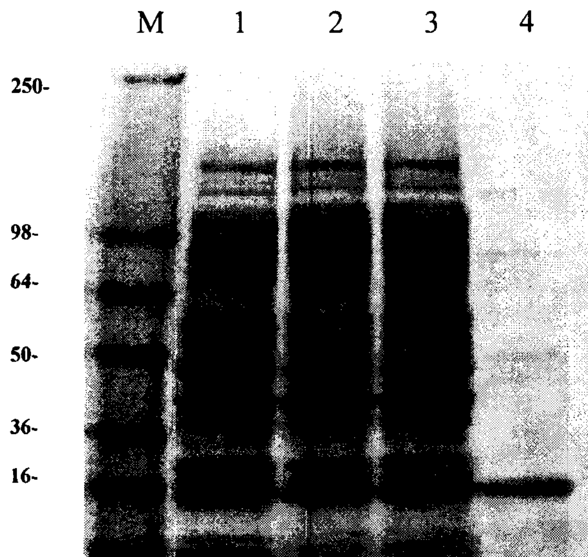

FIGURE 2: SDS-PAGE analysis of recombinant hTIMP-2 expressed by *E.coli* cells. Time course of fermentation: Lane M: prestained molecular weight marker (Invitrogen); lane 1: 14 h fermentation time; lane 2: 23 h fermentation time; lane 3: 38 h fermentation; lane 4: rhTIMP-2 standard.

FIGURE 3

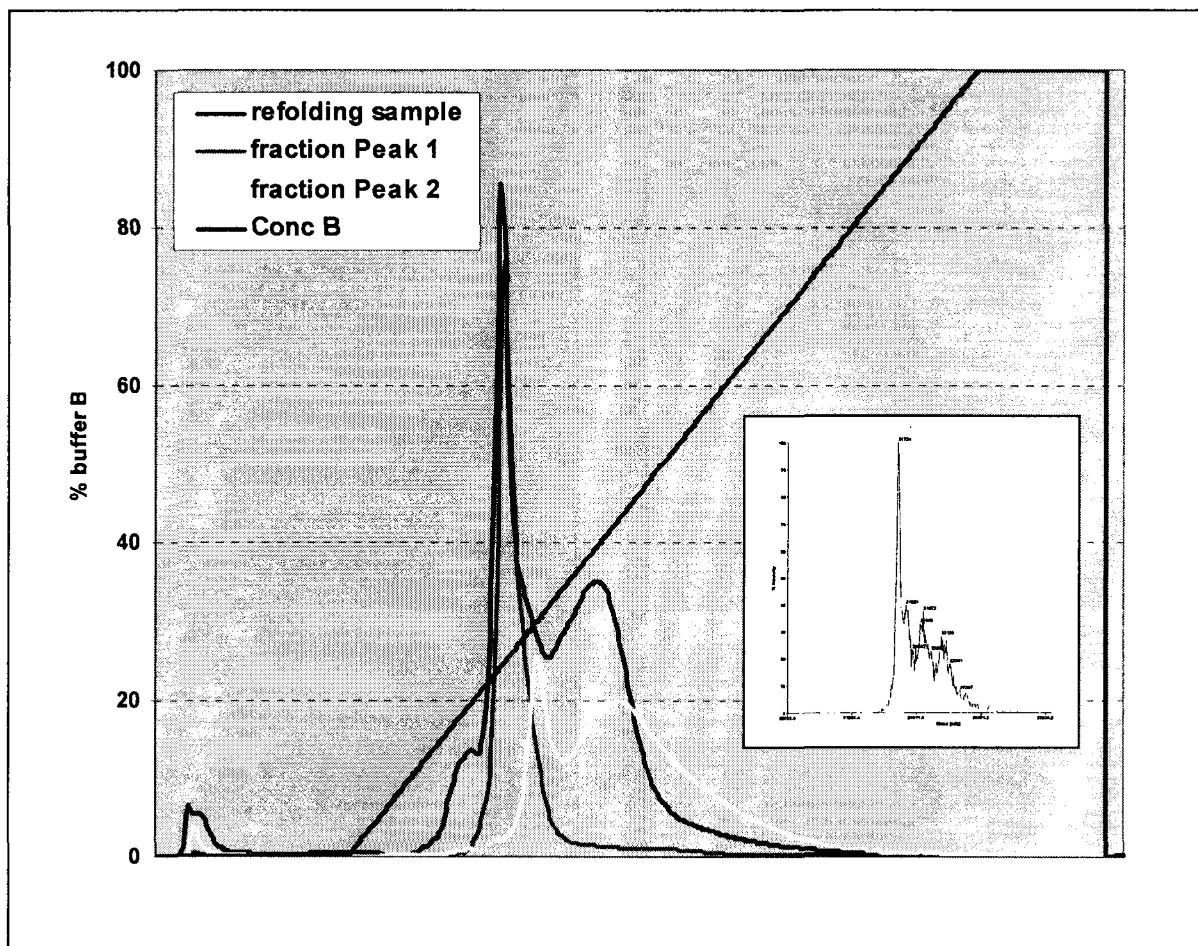

FIGURE 3: Analytical anion exchange chromatogram on a Mono-Q column with the refolded rhTIMP-2 concentrate, the pooled fraction Peak 1 and fraction Peak 2. Insert: MALDI-TOF-MS spectra of rhTIMP-2 in positive ion mode with sinapinic acid as matrix. $[M]_{calc.}$ = 21754.8, $[M]_{found}$ = 21754.

FIGURE 4 A/B

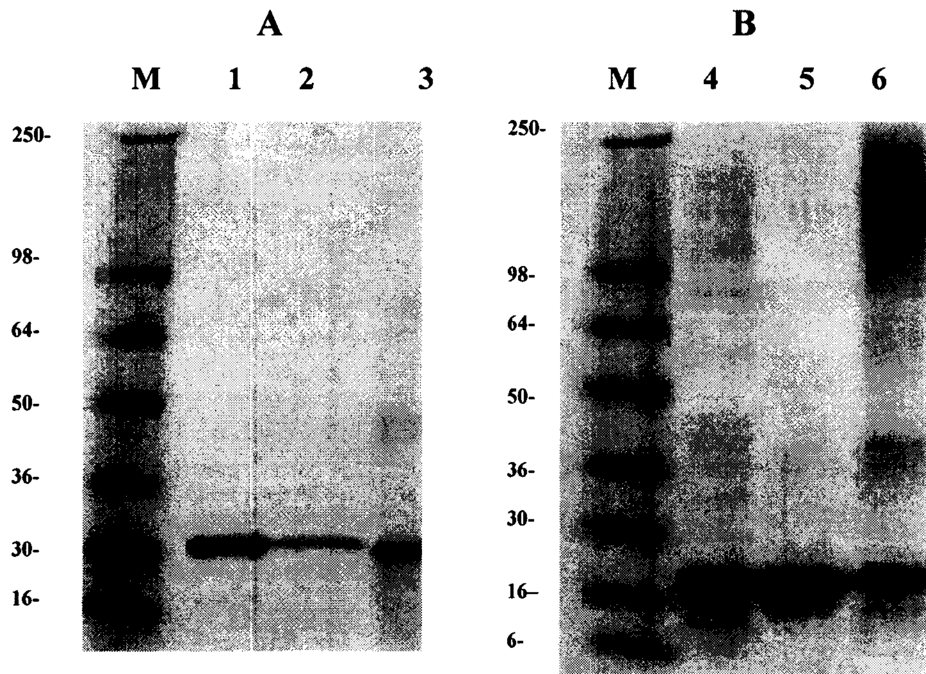

FIGURE 4: SDS-PAGE analysis of refolded, purified rhTIMP-2 after anion exchange chromatography. **A**) reducing condition (with DTT) - lane M: prestained molecular weight marker (Invitrogen); lane 1: fraction Peak 1; lane 2: authentic sample hTIMP-2; lane 3: fraction Peak 2; **B**) non-reducing condition (w/o DTT) - lane M: prestained molecular weight marker (Invitrogen); lane

FIGURE 5

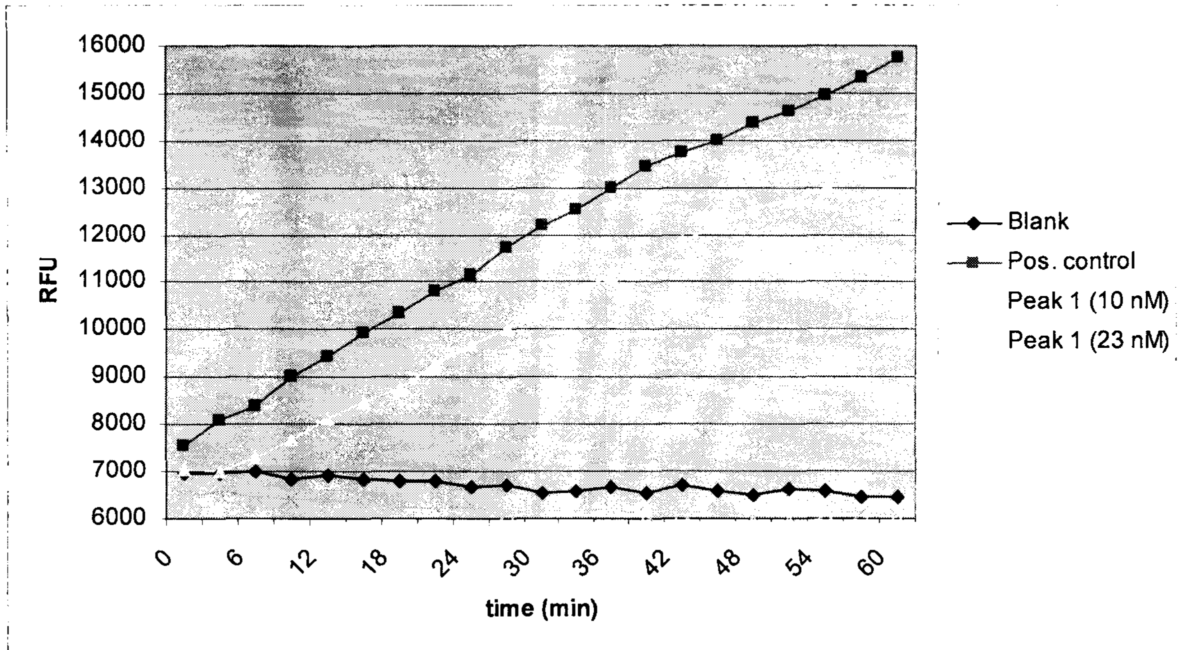

FIGURE 5: Fluorogenic time course assay of inhibition of matrix metalloproteinase 1 (10 nM) by rhTIMP-2 at an inhibitor concentration of 10 and 23 nM, respectively. The reaction was started by the addition the fluorogenic peptide substrate RAE(pNA)LGLPKAbz (10mM). Fluorescence was analyzed in 3 min intervals for 1 h at 37°C.

FIGURE 6

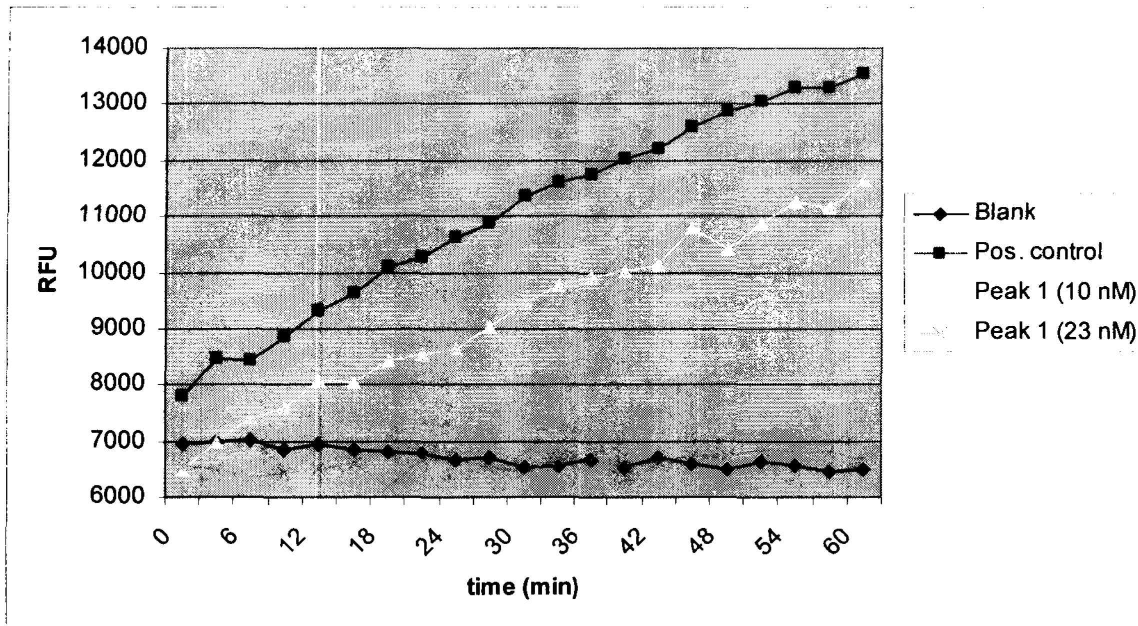

FIGURE 6: Fluorogenic time course assay of inhibition of matrix metalloproteinase 2 (5 nM) by rhTIMP-2 at an inhibitor concentration of 10 and 23 nM, respectively. The reaction was started by the addition the fluorogenic peptide substrate RAE(pNA)LGLPKAbz (10mM). Fluorescence was analyzed in 3 min intervals for 1 h at 37°C.