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A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases

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(7-methoxycoumarin-4-yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) has been synthesised as a fluorogenic substrate for the matrix metalloproteinases. The highly fluorescent 7-methoxycoumarin group is efficiently quenched by energy transfer to the 2,4-dinitrophenyl group. The punctuated metalloproteinase (PUMP, EC 3.4.24.23) cleaves the substrate at the Gly-Leu bond with a 190-fold increase in fluorescence (λ_{ex} 328 nm, λ_{em} 393 nm). In assays of the human matrix metalloproteinases, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 is about 50 to 100 times more sensitive than dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 and continuous assays can be made at enzyme concentrations comparable to those used with macromolecular substrates. Specificity constants (k_{cat}/K_m) are reported for both synthetic substrates with PUMP, collagenase, stromelysin and 72 kDa gelatinase.

Fluorescence quenching; Endopeptidase; Peptide synthesis; Specificity constant

1. INTRODUCTION

Recently, kinetic studies of the matrix metalloproteinases have been aided by the introduction of quenched fluorescent substrates [1,2]. In these substrates a short sequence of amino acids containing the scissile peptide bond separates the naturally fluorescent amino acid tryptophan from a dinitrophenyl (Dnp) group that acts as an internal quencher. Quenching occurs by resonance energy transfer [3] and is almost unaffected by the nature of the intervening amino acids, so that with appropriate sequences some degree of specificity for the individual proteinases can be achieved [2].

The use of tryptophan in quenched fluorescent peptides [1,2,4] is associated with several disadvantages [5]. It is not very fluorescent, so that the assays are quite insensitive, and the solid phase synthesis of tryptophan-containing peptides is complicated by side reactions of the indole ring [6]. Moreover, tryptophan is abundant in proteins, so that crude enzyme preparations or tryptophan-containing inhibitors will contribute a high background fluorescence. We have shown that these problems may be overcome by replacing tryptophan by derivatives of 7-methoxycoumarin, which are highly fluorescent and chemically stable, and also efficiently quenched by the Dnp group [5,7]. We now report the synthesis and characterisation of such a substrate for the matrix metalloproteinases.

Abbreviations: Dnp, 2,4-dinitrophenyl; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; Mca, (7-methoxycoumarin-4-yl)acetyl; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid.

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2. EXPERIMENTAL

2.1. *Peptide synthesis*

Fmoc amino acids and 7-methoxycoumarin-4-acetic acid were from Sigma. [Bis(trifluoroacetoxy)iodo]benzene was prepared from [bis(acetoxy)iodo]benzene (Aldrich) [8]. Pepsyn KB resin and Fmoc-Gly-OPfp were from Milligen/Biosearch. *p*-[(*RS*)-α-[1-(9H-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]Phenoxyacetic acid [9], PyBOP [10] and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 were from Novabiochem. N^{α}-Dnp-lysine hydantoin [11] was synthesised by the method of Stark and Smyth [12]. HPLC analyses were made on a Varian 5000 with a Techopak 10C18 column and an acetonitrile gradient in 0.1% TFA.

2.2. N^2-*Fmoc*-N^3-*Dnp*-L-*2,3-diaminopropionic acid*

Fmoc-Asn-OH (1.42 g, 4.0 mmol) was dissolved in dimethylformamide (10 ml). Water (2.0 ml), pyridine (0.75 ml, 8.0 mmol) and [bis(trifluoroacetoxy)iodo]benzene (2.60 g, 6.0 mmol) were added, and the mixture was stirred at 20°C overnight. The solvents were removed at 50°C in vacuo and the oil remaining was dissolved in water (100 ml) and conc. HCl (10 ml). This solution was extracted with ether (2 × 50 ml) and brought to pH 7 with solid Na_2CO_3. $NaHCO_3$ (0.67 g, 8.0 mmol), ethanol (100 ml) and Dnp-F (0.5 ml, 4.0 mmol) were added, and the mixture was stirred at 20°C for 2 h. Ethanol was removed at 40°C in vacuo and the aqueous solution was brought to pH 1 with HCl. The crude product (1.59 g, 81% yield) was recrystallised from hot ethanol/water. N^2-Fmoc-N^3-Dnp-L-2,3-diaminopropionic acid (M_r 492.45) had m.p. 117–118°C (Found: C, 58.4%; H, 4.2%; N, 11.3%; $C_{24}H_{20}N_4O_8$ requires C, 58.5%; H, 4.1%; N, 11.4%).

2.3. *Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-*NH_2

This was synthesised by the Fmoc-polyamide method [13]. Pepsyn KB resin (1 g), esterified with 0.16 mmol Fmoc-Gly, was deprotected

and treated with *p*-[(*RS*)-α-[1-(9H-fluoren-9-yl)methoxy-formamido]-2,4-dimethoxybenzyl]phenoxyacetic acid (432 mg, 0.8 mmol), PyBOP (416 mg, 0.8 mmol), HOBt (108 mg, 0.8 mmol) and di-isopropylethylamine (0.21 ml, 1.2 mmol). In subsequent steps, Fmoc-amino acids (0.48 mmol) were coupled with PyBOP (0.48 mmol), HOBt (0.48 mmol) and di-isopropylethylamine (0.72 mmol), using a double- cycle protocol. Three couplings of 7-methoxycoumarin-4-acetic acid were necessary to ensure complete reaction. The product was cleaved from the resin with TFA/water (19:1, v/v, 40 ml) for 2 h at 21°C, applied to a column (1.7 cm × 50 cm) of Vydac 218TPB1520, and eluted with a gradient of 5–75% acetonitrile in aqueous 0.1% TFA. Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-$NH_2 \cdot CH_3COOH$ (M_r 1153.2) was freeze-dried from 40% (v/v) acetic acid (yield 112 mg, 61%). Stock solutions were made in dimethylsulphoxide and the concentrations determined from the absorbance at 410 nm, assuming ε=7,500 $M^{-1} \cdot cm^{-1}$, the value measured with N^5-Dnp-lysine hydantoin.

2.4. *Synthesis of Mca-Pro-Leu and Mca-Pro-Leu-Gly*

Pro-Leu methyl ester (1 mmol) [5] in dimethylformamide (2 ml) was treated with 7-methoxycoumarin-4-acetic acid (234 mg, 1 mmol), PyBOP (520 mg, 1 mmol), HOBt (135 mg, 1 mmol) and di-isopropylethylamine (0.7 ml, 4 mmol). After 4 h, chloroform (50 ml) was added and the solution extracted in turn with 10% citric acid (50 ml), 5% $NaHCO_3$ (50 ml) and saturated NaCl (50 ml). The chloroform phase was dried ($MgSO_4$) and evaporated, and the residue was treated with methanol (4 ml) and 1M NaOH (4 ml) for 1 h at 21°C. The mixture was neutralised with 1 M HCl, diluted with water (60 ml) and applied to a column (1.7 × 50 cm) of Vydac. Mca-Pro-Leu was eluted with a gradient of 5–50% acetonitrile in aqueous 10 mM ammonium acetate, pH 5.5 (yield 149 mg, 34%).

A portion of Mca-Pro-Leu was converted to Mca-Pro-Leu-Gly by a similar procedure and purified on Vydac. Amino acid analysis gave the molar ratios: Pro, 0.74; Leu, 1.00; Gly, 1.06.

2.5. *Metalloproteinase preparation and activation*

Human pro-collagenase, pro-stromelysin, pro-PUMP and pro-72 kDa gelatinase were expressed in NSO myeloma cells and purified as described [14,15]. The pro-enzymes were activated immediately prior to use. Pro-collagenase was treated with trypsin (5 μg/ml) and stromelysin (0.4 μg/ml) for 30 min at 37°C, before addition of soybean trypsin inhibitor (50 μg/ml). Pro-stromelysin was treated with trypsin (5 μg/ml) for 30 min at 37°C, followed by soybean trypsin inhibitor (50 μg/ml). 72 kDa gelatinase was activated with 4-aminophenylmercuric acetate (1 mM) for 20 h at 4°C. PUMP was activated at 56°C for 1 h, or by treatment with 4-aminophenylmercuric acetate (1 mM) for 1 h at 37°C.

2.6. *Active site titration*

Enzyme concentrations were determined by active site titration with the stoichiometric inhibitor TIMP-1 [16]. Briefly, collagenase (approx. 250 nM), stromelysin (50 nM) or 72 kDa gelatinase (0.8 nM) was preincubated with various concentrations of TIMP-1 for 4 h at 25°C before 10-fold dilution into assay buffer (0.1 M-Tris-HCl, pH 7.5, containing 0.1 M NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35) containing 1.6 μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Initial velocities were plotted against TIMP-1 concentration and the enzyme concentration determined from the intercept on the ordinate. PUMP titration required higher enzyme concentrations (approx. 500 nM), and dilutions were made into buffer containing Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, from which aliquots were taken at time points up to 30 min into 0.1 M sodium acetate, pH 4.0, to stop the reaction before measurement of the increase in fluorescence.

2.7. *Fluorimetric analyses*

These were made in Perkin-Elmer LS-3 or LS-5B spectrofluorimeters at 37°C (PUMP, stromelysin and collagenase) or 25°C (72 kDa gelatinase). The LS-3 was controlled by an IBM-compatible computer running the FLU-SYS software [17]. In assays with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (λ_{ex} 328 nm, λ_{em} 393 nm), the instruments were set to zero with substrate in assay buffer, and then calibrated with Mca-Pro-Leu so that the full scale deflection corresponded to between 2 and 10% hydrolysis of the substrate (cf. [7]). For each enzyme, the initial rate of cleavage of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, measured over 10 to 15 min, was proportional to substrate concentration in the range 1–8 μM. A concentration of 1.6 μM was used to determine values of k_{cat}/K_m. Stopped assays were made with Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (λ_{ex} 283 nm, λ_{em} 350 nm), in which aliquots were taken at time points up to 2 h into 0.1 M sodium acetate, pH 4.0. For PUMP, collagenase and stromelysin, the initial rate of hydrolysis was proportional to substrate concentration in the range 20–100 μM and a concentration of 20 μM was used for k_{cat}/K_m determinations. For gelatinase, which has a K_m of 70 μM, initial rates obtained in the range 20–150 μM were used to determine the kinetic parameters by non-linear regression [18].

3. RESULTS AND DISCUSSION

3.1. *Design of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2*

Stack and Gray [1] have described the assay of pig synovial collagenase and gelatinase with the quenched fluorescent peptide Dnp-Pro-Leu-Gly+Leu-Trp-Ala-D-Arg-NH_2, in which the symbol "+" represents the scissile bond. We have retained the Pro-Leu-Gly-Leu sequence, but labelled the N-terminus with the highly fluorescent (7-methoxycoumarin-4-yl)acetyl (Mca) group. The quenching Dnp group has been inserted with the novel N^5-Dnp-L-2,3-diaminopropionic acid (Dpa) in the P2′ position [19], as the matrix metalloproteinases favour aromatic side chains at P2′ [2,20].

3.2. *Spectral properties*

In the unquenched peptide Mca-Pro-Leu, the fluorescence has an excitation maximum at 328 nm and the emission is centred around 393 nm (Fig 1). The absorbance maximum is at 324 nm (ε=12,900 $M^{-1} \cdot cm^{-1}$). The absorption spectrum of the Dnp group in Dpa is assumed to be identical to that in the homologous N^5-Dnp-lysine hydantoin (Fig. 1), which has a maximum at 363 nm (ε=15,900 $M^{-1} \cdot cm^{-1}$) and a prominent shoulder at 410 nm (ε=7,500 $M^{-1} \cdot cm^{-1}$). This shoulder

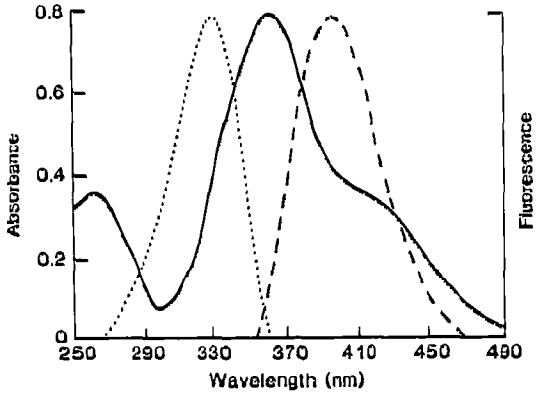

Fig. 1. Spectral overlap of the Dnp group and Mca-Pro-Leu. The absorption spectrum of 50 μM Dnp-lysine hydantoin (—) and the fluorescence excitation (····) and emission (----) spectra of 1 μM Mca-Pro-Leu were recorded in assay buffer.

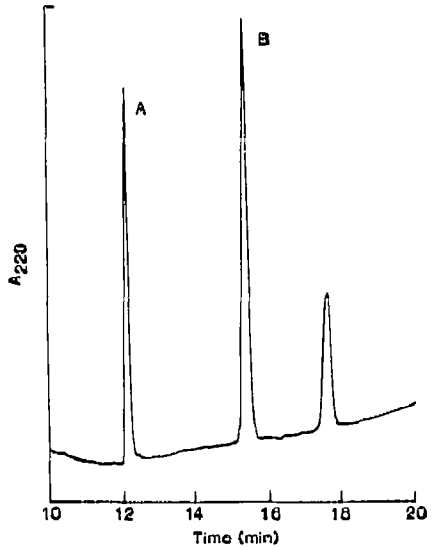

Fig. 2. HPLC analysis of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 hydrolysis by PUMP. The chromatograph shows the unhydrolysed substrate (17.6 min) and the two fragments (A: Leu-Dpa-Ala-Arg-NH_2, 12.0 min; B: Mca-Pro-Leu-Gly, 15.3 min) produced by PUMP.

overlaps the emission spectrum of Mca, as is required for efficient energy transfer [3]. When the fluorescence emissions were compared at a concentration of 1 μM, Mca-Pro-Leu was 130 times more fluorescent than Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2.

3.3 *Cleavage of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 by PUMP*

When the substrate (20 μM) was incubated with PUMP (0.2 μg) in assay buffer at 37°C, two new peaks (A and B) were observed by HPLC (Fig. 2). Peak B had the same retention time as Mca-Pro-Leu-Gly, showing that cleavage had occurred, as expected [1,2], at the Gly-Leu bond. When the hydrolysis of the substrate (1 μM) by PUMP (0.8 μg) was followed to completion over 30 min in the fluorimeter, the fluorescence increased 190-fold (data not shown), so the degree of quenching must be close to 99.5%. In continuous assays made with 10 μM substrate, hydrolysis was readily detectable with PUMP concentrations less than 0.2 nM (Fig. 3). By contrast, the fluorescence increased only about 25-fold during the cleavage of Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2, and no activity could be seen in assays with this substrate at enzyme concentrations less than 1 nM (Fig. 3).

Table I

Specificity constants for hydrolysis of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 by PUMP, collagenase, stromelysin and 72 kDa gelatinase. Values are the means of six determinations.

	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	
Enzyme	Mca-P-L-G-L-Dpa-A-R	Dnp-P-L-G-L-W-A-r
PUMP[a]	169,000	11,700
Collagenase[a]	14,800	830
Stromelysin[a]	23,000	2,200
Gelatinase[b]	629,000	58,000

[a]37°C; [b]25°C

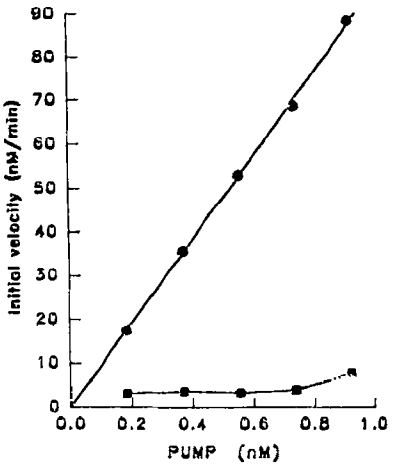

Fig. 3. Standard curves for the continuous assay of PUMP with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (●) and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (■). Initial rates of product formation were measured with different amounts of PUMP/assay.

3.4. *Comparisons with other matrix metalloproteinases*

Values of the specificity constant k_{cat}/K_m were determined with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 for PUMP, collagenase, stromelysin and 72 kDa gelatinase (Table I). In all cases, much higher values of k_{cat}/K_m were observed with the new substrate. If we assume the stereoisomerism at P4′ to be unimportant, the two substrates differ only at P4 and P2′ and the increased values of k_{cat}/K_m must reflect more favourable interactions at these subsites. The specificity studies of Netzel-Arnett et al. [20] suggest that the P2′ site may be the more important one, in which case the increased specificity results from the replacement of tryptophan by Dpa. The more favourable binding of Dpa probably reflects the nature of the N^3-Dnp group, which although predominantly hydrophobic, has also a dipolar character due to resonance effects [21], which may lead to additional hydrogen bonding interactions in the P2′ pocket.

3.5. *Assay sensitivity*

Our assays were made with substrate concentrations well below K_m to avoid absorptive quenching effects. When $[S] \ll K_m$, the minimum enzyme concentration $[E]_{min}$ producing a measurable velocity v_{min} at a constant substrate concentration [S] can be estimated from equation (1):

$$[E]_{min} = v_{min}/(k_{cat} \cdot [S]/K_m).$$

When assays made with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 were compared, those made with the new substrate were more sensitive than could be accounted for by the differences in k_{cat}/K_m, presumably because Mca is more fluorescent than tryptophan. Thus continuous assays with Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 could be made routinely with 100 pM PUMP, 10 nM collagenase, 3 nM stromelysin and 50 pM gelatinase. Approximately 50- to 100-fold higher enzyme concentrations were required in assays with Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2

4. CONCLUSION

Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 is the most sensitive substrate so far described for continuous assays of the matrix metalloproteinases. Not only are the values of k_{cat}/K_m higher than those reported previously, but the cleavage product is more readily detectable, as the Mca group is both more fluorescent than tryptophan and more efficiently quenched by the Dnp group. With the exception of collagenase, continuous assays can be made with the new substrate at enzyme concentrations similar to those required in assays with their preferred macromolecular substrates. Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 is suitable therefore for the assessment of total matrix metalloproteinase activity in crude preparations. Further work to develop peptides that are specifically cleaved by the individual enzymes will permit their measurement under these conditions.

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