Biochem. J. (1989) 260, 259–263 (Printed in Great Britain)

A continuous fluorimetric assay for clostridial collagenase and Pz-peptidase activity

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The peptide derivative N^{α}-(2,4-dinitrophenyl)-L-prolyl-L-leucyl-glycyl-L-prolyl-L-tryptophanyl-D-lysine (Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys) has been found to be a convenient substrate for the assay of clostridial collagenase and Pz-peptidase. The substrate shows a 25-fold enhancement of fluorescence ($\lambda_{ex.}$ 283 nm, $\lambda_{em.}$ 350 nm) following hydrolysis of the Leu^2–Gly^3 peptide bond. The value of K_m for clostridial collagenase was 17 μM. The substrate for the first time makes possible continuous fluorimetric assays for Pz-peptidase and clostridial collagenase.

INTRODUCTION

The collagenase of *Clostridium histolyticum* (EC 3.4.24.3) is commonly assayed with the 'Pz-peptide', Pz-Pro-Leu-Gly-Pro-D-Arg, a synthetic peptide with an amino-acid sequence based on the -Gly-Pro-Xaa- tripeptide repeating pattern of the helical region of collagen (Wünsch & Heidrich, 1963). Spectrophotometric substrates that show a very small change in absorbance in continuous assays have also been described (Steinbrink *et al.*, 1985), but these require sophisticated equipment, and practical considerations limit most experimenters to the use of assays with collagen or the Pz-peptide.

Although the Pz-peptide is not a substrate for mammalian collagenase, the tissues of birds and mammals do contain enzymes that hydrolyse the substrate, and these have been called Pz-peptidases (Hino & Nagatsu, 1976; Morales & Woessner, 1977; Lessley & Garner, 1985). Pz-peptidase activity tends to rise in biological situations in which collagen degradation is accelerated, leading to the suggestion that Pz-peptidases play a part in the late stages of the degradation of collagen (Morales & Woessner, 1977; Chikuma *et al.*, 1985). Because of this, there is considerable interest in the biochemistry of these enzymes.

It can be seen that the Pz-peptide is an important substrate for work with clostridial collagenase and the Pz-peptidases, but there are serious practical limitations to its use. The product of the hydrolysis of the peptide, Pz-Pro-Leu, has similar spectral properties to the substrate, so assays involve the separation of the product from the unhydrolysed substrate by extraction into ethyl acetate (Wünsch & Heidrich, 1963) or by h.p.l.c. (Chikuma *et al.*, 1985; Biondi *et al.* 1988). It is not possible for workers using this substrate to benefit from the many advantages of continuous kinetic assays.

For these reasons, there is a need for a new assay for clostridial collagenase and Pz-peptidase. We have found that good results can be obtained by use of a 'quenched fluorescence' substrate structurally related to the Pz-peptide. The principle of quenched fluorescence assays has been reviewed by Yaron *et al.* (1979). Briefly, the substrate contains a potentially fluorescent group but also a group that quenches the fluorescence, so that the uncleaved substrate has little or no fluorescence. Cleavage of the substrate separates the two groups, and fluorescence appears.

EXPERIMENTAL

Materials

Materials for peptide synthesis. Pepsyn KB resin and Fmoc-amino acid reagents were from MilliGen. Dnp-Pro was made as described by Porter & Sanger (1948). Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate ('BOP') was from Novabiochem. Dimethylformamide (Fisons) was treated with molecular sieve 4A and redistilled under reduced pressure before use. Trifluoroacetic acid (Fluka) was redistilled. Vydac C_{18} was from Technicol.

Other materials. Pz-Pro-Leu-Gly-Pro-D-Arg, Ac-Trp-NH_2 and collagenase from *Clostridium histolyticum* (Types I and VII) were obtained from Sigma. Pz-Pro-Leu was from Fluka.

Pz-peptidase from rabbit muscle was prepared as described by Tisljar & Barrett (1989). Pz-peptidase from chicken liver was purified by a procedure (A. J. Barrett, M. A. Brown & U. Tisljar, unpublished work) involving anion-exchange chromatography, gel permeation chromatography, copper-chelate chromatography and chromatofocusing. The specific activity of the preparation on Pz-peptide was approximately similar to that from chick embryos described by Morales & Woessner (1977).

Synthesis of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys

This was synthesized step-wise from the *C*-terminus by the Fmoc-polyamide method using standard procedures (Atherton *et al.*, 1981) on a Cambridge Research Biochemicals Pepsynthesiser (cf. Dryland & Sheppard, 1986). Pepsyn KB resin (1.0 g, capacity 0.1 mmol) was treated with the symmetrical anhydride (0.5 mmol) of Fmoc-D-Lys(Boc) in the presence of 4-dimethylamino-

Abbreviations used: Boc, *tert*-butyloxycarbonyl; Dnp, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethyloxycarbonyl; Pz, phenylazobenzyloxycarbonyl.

pyridine (0.1 mmol). Subsequent Fmoc amino acids were coupled as their pentafluorophenyl esters (0.5 mmol) in the presence of 1-hydroxybenzotriazole (0.5 mmol). Dnp-Pro (0.5 mmol) was coupled with BOP (Le-Nguyen *et al.*, 1987) in the presence of di-isopropylethylamine (0.5 mmol of each). At the completion of the synthesis, the resin was gently shaken for 15 min with a mixture of trifluoroacetic acid (55 ml), anisole (3 ml) and ethanedithiol (2 ml) to remove the Boc protecting group. The resin was washed and dried (Atherton *et al.*, 1981), and the peptide was released by treatment with 5 ml of 1 M-NaOH at 0 °C for 15 min. Acetic acid (10% v/v, 5 ml) was added and the resin was washed with water until the washings were colourless. The pooled washings were filtered and applied to a column (15 mm × 440 mm) of Vydac C_{18} (15–20 μm). The product was eluted using a gradient of 5–50% acetonitrile in water containing 10 mM-ammonium acetate, pH 5.5 (Jackson & Young, 1987).

The peptide showed a single peak on analytical h.p.l.c. in solvent systems A and B (see below) (Fig. 3). The amino acid composition was: proline 1.51, glycine 0.93, leucine 1.00 and lysine 0.90 mol/mol. We consider this to represent satisfactory agreement with expectation, taking account of the expected partial hydrolysis of the Dnp-Pro bond. A 1 mM stock solution of the substrate in water was stored at 4 °C.

Synthesis of Dnp-Pro-Leu

This was made by the method of Porter & Sanger (1948) and purified by reverse phase chromatography on Vydac C_{18}, as described above. The compound was homogeneous in h.p.l.c. in systems A and B.

High performance liquid chromatography

This was done on a Varian LC5000 instrument equipped with the Vista 402 data processing system, and a Zorbax ODS (4.6 mm × 250 mm) column. The solvent systems used for separation of peptide derivatives were system A: acetonitrile/water/trifluoroacetic acid (linear gradient from 5 to 100% acetonitrile in water, both components containing 0.1% trifluoroacetic acid), and system B: acetonitrile/water/acetic acid/triethylamine (linear gradient from 5 to 100% acetonitrile in 10 mM-acetic acid adjusted to pH 5.8 with triethylamine).

Amino acid analysis

Peptides were hydrolysed in 6 M-HCl containing 1% phenol at 110 °C during 24 h. The amino acids were then subjected to pre-column derivatization with 9-fluorenylmethyl chloroformate, and separated by h.p.l.c. as described by Cunico *et al.* (1986). Alternatively, amino acid analyses were obtained commercially (Cambridge Research Biochemicals).

Fluorimetric analyses

These were made in a Perkin–Elmer LS-3 spectrofluorimeter linked to an Olivetti M-24 computer running software for collection and analysis of the data (the FLU system of A. J. Barrett & N. D. Rawlings, unpublished work). The content of the fluorimeter cuvette (quartz) was stirred and maintained at 40 °C during the experiments. When necessary, the temperature of the cell contents was determined by use of a thermistor probe. The instrument was calibrated (at 40 °C) to read 1000 units of fluorescence with 1.0 μM-Ac-Trp-NH_2, with $\lambda_{ex.}$ 283 nm and $\lambda_{em.}$ 350 nm. One fluorescence unit then corresponded to formation of 1 nM-product in 2.5 ml by hydrolysis of this concentration of substrate, and results have been expressed in this way.

For continuous assays, 2.45 ml of assay buffer (50 mM-Tris/HCl, pH 7.8, 0.05% Brij-35 and 10 mM-$CaCl_2$, also containing 5 mM-2-mercaptoethanol for Pz-peptidase) prewarmed to 40 °C was pipetted into the fluorimeter cuvette, followed by 25 μl of enzyme sample. After 3 min, the reaction was started by the introduction of 25 μl of substrate stock solution (1 mM), and the fluorescence was recorded every 1 s. The rate of increase in fluorescence over a suitable time interval (10–30 min) was calculated by linear regression analysis of the values against time.

For fixed time assays, reaction mixtures made up as above, but in glass tubes, were incubated for 20 min in a waterbath at 40 °C before the reaction was stopped by the addition of 100 μl of 2 M-sodium formate buffer, pH 3.7. The fluorescence of the samples (still at 40 °C) was measured as above. (The fluorescence is strongly temperature-dependent; see below.)

RESULTS

Temperature-dependence of fluorescence of Gly-Pro-Trp-D-Lys

We have observed that some but not all fluorophores show strong negative temperature dependence for fluorescence emission. This can give rise to inaccuracies in fluorimetry if sample temperature is not accurately controlled. Fig. 1 shows the effect of temperature on the fluorescence of the product of hydrolysis of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys by clostridial collagenase. The product, which is Gly-Pro-Trp-D-Lys (see below) was in solution in the assay buffer (see the Experimental section).

The reference standard used for calibration of the fluorimeter, Ac-Trp-NH_2, showed a similar effect of temperature. Controls showed that the loss of fluorescence with raised temperature was reversible: there was no significant destruction of the fluorophore during the experiments.

Continuous assay for clostridial collagenase

Assays were made as described in the Experimental section, with 0, 0.625, 1.25, 1.875, 2.5 and 3.125 μg of clostridial collagenase (Type I). The rates of increase in fluorescence were plotted (Fig. 2*a*), and showed a linear relationship between enzyme concentration and rate of increase in fluorescence. The sensitivity of the assay for the pure enzyme is actually much greater than this, since the chromatographically purified commercial Type VII material showed 15-fold greater specific activity, but the reaction rates tended to fall off with time, presumably as a result of spontaneous denaturation of the enzyme in very dilute solution. A set of replicate assays ($n = 7$) for 1.6 μg of Type I collagenase gave results with a standard deviation of 7.8% of the mean.

Discontinuous assay

The results of 'stopped' assays made as described in the Experimental section, for 0, 0.625, 1.25, 1.875, 2.5 and 3.125 μg of the crude clostridial collagenase/tube are shown in Fig. 2(*b*). Again, a linear dose–response curve was obtained. The results of a set of replicate assays ($n = 10$) with 1.6 μg of collagenase showed a standard deviation of 5.8% of the mean.

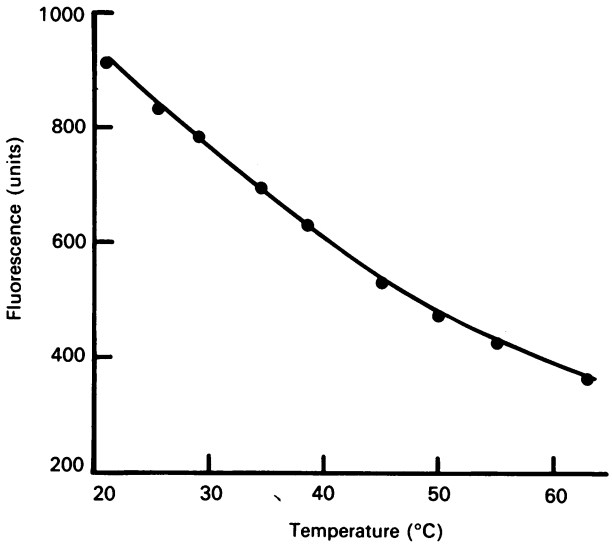

Fig. 1. Temperature-dependence of fluorescence of Gly-Pro-Trp-D-Lys

The substrate (10 μM in assay buffer) was completely hydrolysed by 5 μg of Type I collagenase during 4 h at 40 °C. The effect of temperature on the fluorescence (arbitrary units) of the resulting solution was then determined.

K_m of clostridial collagenase for Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys

Rates of hydrolysis of the new substrate by clostridial collagenase (Type I) were measured at [S] values in the range 4–50 μM, and the results were fitted to the Michaelis–Menten equation by non-linear regression. The K_m value obtained was 17.3 ± 2.8 (S.E.) μM.

Continuous assay for Pz-peptidase activity

Purified rabbit Pz-peptidase also gave satisfactory results in both continuous and discontinuous assays. The enzyme was activated during 3 min preincubation with 5 mM-2-mercaptoethanol (Tisljar & Barrett, 1989). The results of the continuous assay with 0.35, 0.70, 1.05, 1.40 and 1.75 μg of the rabbit enzyme preparation are shown in Fig. 2(*c*). Chicken Pz-peptidase has also been satisfactorily assayed by this method (results not shown).

Identification of bond cleaved in the substrate

A 10 μM solution of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys in collagenase assay buffer (see above) was treated with collagenase (5 μg/ml) at 40 °C until there was no further increase in fluorescence (4 h). During this period, the fluorescence of the solution (10-fold diluted for measurement) increased by 25-fold. Samples of the solution before and after the enzyme treatment were run in h.p.l.c. in system B, the effluent being monitored at 220 and 350 nm in separate runs. Incubation with the enzyme resulted in the formation of two new peaks (A and B) at 220 nm (Fig. 3). Peak B was also detectable at 350 nm, and had an elution time identical with that of standard Dnp-Pro-Leu. Effluent fractions were collected, and those comprising the new peaks were taken down to dryness under reduced pressure and hydrolysed for amino acid analysis. It was found that peak A contained approximately equimolar amounts of glycine, proline and lysine, but no leucine, whereas peak B contained proline and

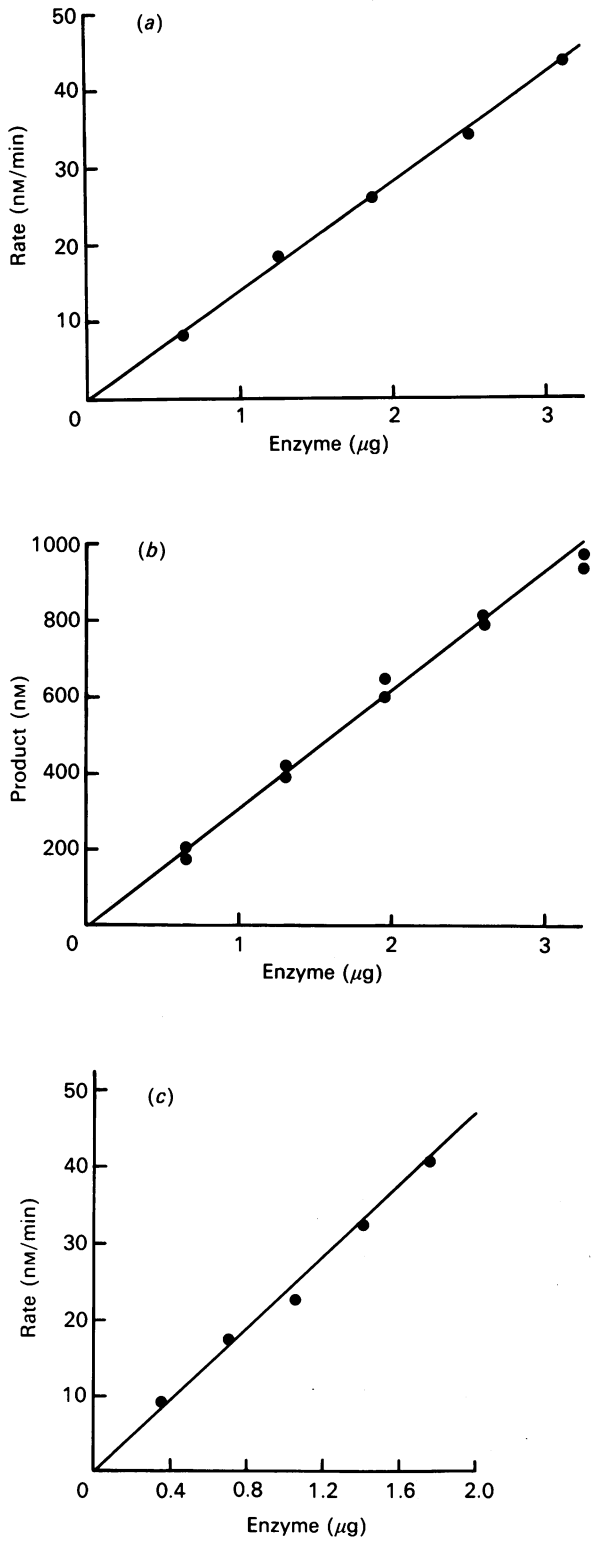

Fig. 2. Standard curves for continuous and discontinuous assays

(*a*) Continuous assay of clostridial collagenase, (*b*) discontinuous assay of clostridial collagenase, and (*c*) continuous assay of rabbit muscle Pz-peptidase activity. In the continuous assays, rates of increase in product concentration were measured with various amounts of enzymic activity/assay. The results shown for the discontinuous assay represent the final concentrations of product after 20 min incubation.

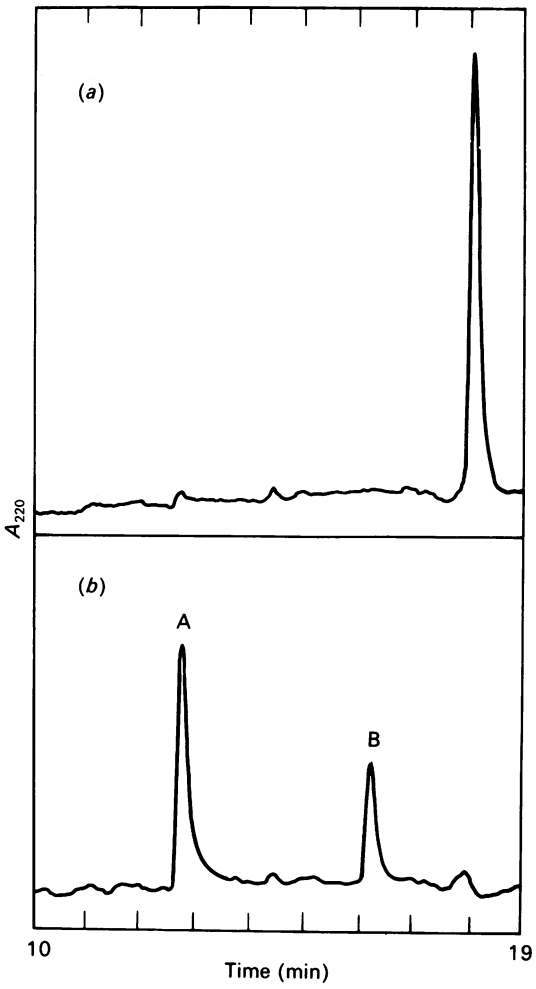

Fig. 3. H.p.l.c. analysis of Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys hydrolysis by clostridial collagenase

The chromatograph was run as described in the Experimental section, and the effluent was monitored at 220 nm. The part (10–19 min) of the elution profiles containing (*a*) the unhydrolysed peptide (elution time 18.0 min), and (*b*) the products of complete hydrolysis of the substrate by clostridial collagenase (A: Gly-Pro-Trp-D-Lys, elution time 12.8 min, and B: Dnp-Pro-Leu, elution time 16.1 min), are shown.

leucine, but no glycine or lysine. As expected, tryptophan was not detected after the acid hydrolysis. We concluded that peak A represented Gly-Pro-Trp-D-Lys and peak B Dnp-Pro-Leu, and that clostridial collagenase cleaves the substrate only at the Leu^2–Gly^3 bond. The same products were seen with Pz-peptidase.

DISCUSSION

We have described the synthesis and application of a quenched fluorescence substrate for clostridial collagenase and Pz-peptidases. The substrate is structurally related to the Pz-peptide, Pz-Pro-Leu-Gly-Pro-D-Arg, but yields a fluorescent product on proteolytic cleavage. In the substrate, Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys, tryptophan is a fluorescent energy donor, the excited state energy of which is transferred to the dinitrophenyl acceptor group by dipole–dipole interactions. The efficiency of quenching in such a system falls off with the sixth power of the distance between donor and acceptor, and so is critically dependent on their separation (Stryer, 1978). The 25-fold enhancement of fluorescence resulting from cleavage of the substrate allows the activities of the enzymes to be monitored either continuously or discontinuously. The discontinuous assays are more convenient than those with the Pz-peptide, but the continuous assays represent the major advantage of the new substrate. At high purity, collagenase and Pz-peptidases tend to be unstable, so that it cannot safely be assumed that rates are steady throughout the whole incubation period for stopped assays. Such problems are at least detected, and potentially solved, in continuous assays.

The fluorescence of both Ac-Trp-NH_2 and Gly-Pro-Trp-D-Lys shows strong negative temperature dependence; this has been characterized for the former compound by Lakowicz & Balter (1982). Because of this, it is important that the fluorimeter be standardized with a reference solution at the same temperature as the samples.

The sensitivity of the new assays is at least as good as that of those with the Pz-peptide. Thus, Wünsch & Heidrich (1963) used up to 20 μg of crude collagenase in 15 min assays, which may be compared with our use of up to 3 μg of a similar preparation over 20 min. Working with the chromatographically purified enzyme, Rajabi & Woessner (1984) used up to 0.25 μg, and we used up to 0.1 μg in our own assays. With purified chicken Pz-peptidase, Morales & Woessner (1977) obtained good absorbance changes with 0.3 μg/assay, and we would use up to 0.1 μg in ours.

A limitation of the assays with Dnp-Pro-Leu-Gly-Pro-Trp-D-Lys is the high blank value seen with crude enzyme samples containing high protein concentrations and therefore significant amounts of unquenched tryptophan. Still better results might be obtained with a substrate containing a fluorophore active at higher wavelengths, if it were suitably quenched. Nevertheless, the present assay has greatly facilitated the isolation and characterization of Pz-peptidases from rabbit muscle (Tisljar & Barrett, 1989) and chicken liver (A. J. Barrett, M. A. Brown & U. Tisljar, unpublished work).

We thank Mr. Simon Wynne for skilled assistance with the peptide synthesis, and the Wellcome Trust for support of U.T.

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Received 17 November 1988/6 January 1989; accepted 9 January 1989