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SUBSTRATE SPECIFICITY OF VERTEBRATE COLLAGENASE

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Summary

Substrate specificity of purified tadpole collagenase (EC 3.4.24.3) has been studied using eleven synthetic peptides. A pentapeptide, *t*-butyloxycarbonylprolylalanylglycylisoleucylalanine amide, was susceptible to the action of the enzyme and an octapeptide, acetylprolylglutaminylglycylisoleucylalanylglycylglutaminylarginine ethyl ester, was proposed to be the best substrate for vertebrate collagenase among the peptides tested.

Vertebrate collagenase (EC 3.4.24.3) is a tissue protease which degrades native and denatured collagen. The enzyme splits at a specific locus on the collagen molecule, three-quarters of the distance from the N-terminus [1]. Recent studies on the sequence of neighbouring peptides released at the initial split [2,3] and of the main body of the α_1 chain [4] have shown that collagenase splits the Gly-Ile bond in the sequence -Thr-Pro-Gly-Pro-Gln-Gly^{772}-Ile^{773}-Ala-Gly-Gln-Arg-Gly- of the main body. The amino acid sequences at the NH_2-terminal portions of the collagenase-digested smaller fragments derived from Type II and III collagens were determined to be Ile-Ala-Gly-Gln-Arg and Leu-Ala-Gly-Leu-Arg, respectively [5]. Therefore, syntheses of peptides having the same or closely similar sequences to that around the side of cleavage were undertaken in order to identify the simplest synthetic peptide substrate and to investigate the enzymatic properties of collagenase.

Synthetic peptides were prepared by classical solution procedures. Purified tadpole collagenase (540 units/ml, specific activity, 3000 units/mg,

*Abbreviations Ac, acetyl; Boc, *t*-butyloxycarbonyl; OEt, ethyl ester; Pyr, pyroglutamyl. All amino acids are L-configuration.

assuming 1 absorbance unit at 280 nm = 1 mg protein/ml) was prepared, as described previously [6,7]. 1 unit of enzyme activity is defined as that activity which decomposes 1 μg of collagen per min at pH 7.5 and 37°C [7]. 250—500 μg of each synthetic peptide, dissolved in 50 μl of 0.05 M Tris·HCl buffer, pH 7.3, containing 0.15 M NaCl and 5 mM $CaCl_2$, was incubated with 10 μl of the enzyme in the same buffer at 37°C. Aliquots of the reaction mixture were withdrawn at various times of incubation, and the rates of hydrolysis were determined by paper electrophoresis using 0.1 M pyridine/0.1 M acetic acid buffer, pH 4.75, at 0.8 mA/cm for 1 h. The reaction products were located by chlorination of the peptide bonds with *t*-butyl hypochlorite followed by treatment with the starch-iodide reagent [8].

A typical pattern of hydrolysis of Ac-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-OEt by collagenase is shown in Fig. 1, where the substrate peptide was com-

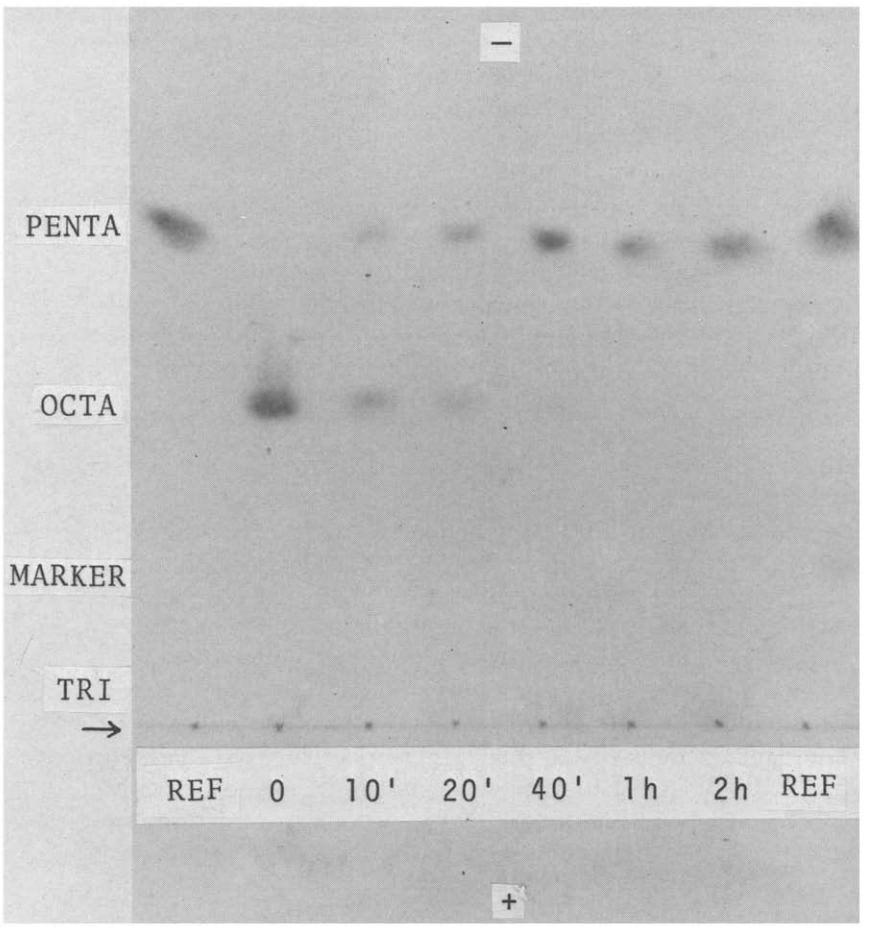

Fig. 1. Electrophoretic pattern of hydrolysis of Ac-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-OEt by purified tadpole collagenase. TRI, Ac-Pro-Gln-Gly-OH; REF and PENTA, H-Ile-Ala-Gly-Gln-Arg-OEt; OCTA, substrate peptide; MARKER, indigotetrasulfonic acid. The small arrow indicates the sample origins. Electrophoresis conditions: buffer: 0.1 M pyridine/acetic acid, pH 4.75; current, 0.8 mA/cm for 1 h; stain: *t*-butyl hypochlorite-starch-potassium iodide.

TABLE I

RELATIVE RATES OF HYDROLYSIS OF SYNTHETIC PEPTIDES BY PURIFIED TADPOLE COLLAGENASE AT pH 7.3

	Peptide	Rate of hydrolysis	
		Percent hydrolysis	Incubation time (h)
I	Ac-Pro-Gly-Pro-Gln-Gly↓Ile-Ala-Gly-Gln-Arg-OEt	100	within 1
II	Ac-Pro-Gln-Gly↓Ile-Ala-Gly-Gln-Arg-OEt	100	within 1
III	H-Pro-Gln-Gly↓Ile-Ala-Gly-Gln-Arg-OEt	less than 50	after 1
IV	Pyr-Gly-Ile-Ala-Gly-Gln-Arg-OEt	0	after 4
V	Ac-Pro-Ala-Gly↓Ile-Ala-Gly-Gln-Arg-OEt	approx. 70–80	after 1
VI	Ac-Pro-Gln-Gly↓Ile-Ala-Gly-OEt*	approx. 50	after 1
VII	Boc-Pro-Gln-Gly↓Ile-Ala-Gly-NH_2	less than 50	after 1
VIII	Boc-Gln-Gly-Ile-Ala-Gly-NH_2*	0	after 4
IX	Boc-Pro-Ala-Gly↓Ile-Ala-NH_2*	less than 50	after 1
X	Boc-Pro-Ala-Gly-Ile-Ala-OH	0	after 4
XI	Boc-Pro-Ala-Gly-Ile-NH_2	0	after 4

*Dissolved in 20% ethanol in 0.05 M Tris·HCl buffer, pH 7.3, containing 0.15 M NaCl and 5 mM $CaCl_2$.

pletely degraded within 1 h incubation. N-terminal amino acid residues released by enzyme digestion were analyzed by the dinitrophenylation method [9]. Only Ile was found in this position as a new N-terminus. The relative rates of hydrolysis of the eleven peptides tested are summarized in Table I, where the susceptible bond is indicated by the small arrow. These results suggest that a minimum size of peptide substrate for collagenase is a pentapeptide of the sequence: Boc-Pro-Gln-Gly-Ile-Ala-NH_2 in which Gln can be replaced by other amino acids such as Ala, as in peptide V or IX. Masking of the N-terminal prolyl residue significantly increased rate of hydrolysis, as indicated by comparison of peptides II and III. Peptides in which this prolyl residue was acetylated were more susceptible to hydrolysis than the *t*-butyl-oxycarbonyl derivatives, as shown by the results with peptides VI and VII. From the observations discussed above and the comparisons of relative rates of hydrolysis of peptides listed in Table I, it seems most likely that Ac-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-OEt (peptide II) is the most convenient synthetic substrate for vertebrate collagenase, combining both small size and maximum susceptibility. Studies on synthetic peptide substrates applicable to the quantitative assay of the enzyme are now in progress.

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