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Synthetic Substrates for Vertebrate Collagenase

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INTRODUCTION

Studies on the substrate specificity of vertebrate collagenase in our laboratories, using peptides having the same or closely similar sequences to that around the cleavage site of the collagen molecule by collagenase, suggested that the enzyme preferentially hydrolyzes the peptide, Ac–Pro–Gln–Gly–Ile–Ala–Gly–Gln–Arg–OEt, at the Gly–Ile bond (1).[2] Therefore, attempts to prepare a synthetic substrate applicable to the quantitative assay for collagenase activity were undertaken.

This paper describes the susceptibility of seven kinds of DNP-peptides to the action of purified tadpole collagenase and human serum peptidase(s), and the application of the most favorable peptide to the assay of collagenase activity in synovial fluids from patients with osteo- and rheumatoid arthritis.

METHODS

Preparation of Seven DNP-Peptides

The following seven DNP-peptides were synthesized by combining stepwise elongation and fragment condensation methods.

I. DNP–Pro–Leu–Gly–Ile–Ala–Gly–Arg–NH_2
II. DNP–Leu–Gly–Ile–Ala–Gly–Arg–NH_2
III. DNP–Pro–Gln–Gly–Ile–Ala–Gly–Gln–D–Arg–OH
IV. DNP–Gln–Gly–Ile–Ala–Gly–Gln–D–Arg–OH
V. DNP–Pro–Gln–Gly–Ile–Ala–Gly–Arg–NH_2
VI. DNP–Pro–Leu–Gly–Ile–Ala–Gly–Gln–D–Arg–OH
VII. DNP–Pro–Gln–Gly–Ile–Ala–Gly–Gln–Arg–NH_2

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[2] Abbreviations used are: Ac, acetyl; DNP, 2,4-dinitrophenyl; OEt, ethyl ester; Ala, alanyl; Arg, arginyl; Gln, glutaminyl; Gly, glycyl; Ile, isoleucyl; Leu, leucyl; and Pro, prolyl. All amino acids are L-configuration except for D-arginine.

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The details of the procedure will be described elsewhere. The analytical data and physical constants for these peptides are listed in Table 1. The purity of all compounds was checked by thin-layer chromatography on plates of Merck–Kieselgel G (Type 60).

The molecular extinction coefficients at 365 nm of Peptide I in ethyl acetate and Peptide III in ethyl acetate–*n*-butanol (1:0.15, v/v) were estimated to be 1.76×10^4 and 1.73×10^4, respectively.

Collagenase

Purified tadpole collagenase was prepared from cultured media of tadpole skin explants by affinity chromatography followed by gel chromatography, as described previously (2,3).

Human Serum Peptidase(s)

Fresh adult human serum (5 ml) was chromatographed on a calibrated Sephadex G-200 superfine gel column equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, containing 0.15 M NaCl and 5 mM $CaCl_2$ (Tris–NaCl–$CaCl_2$) and the effluent fractions corresponding to molecular weights of 100,000–180,000 were pooled and employed as an enzyme source.

Determination of Hydrolysis of Synthetic Peptides

DNP–peptides were dissolved in Tris–NaCl–$CaCl_2$ buffer containing 0.02% bovine serum albumin to make a concentration of 5×10^{-4} M. Each of the peptides (0.1 ml) was mixed with an equal volume of collagenase (6.2 U/ml) or serum peptidase and incubated for various periods of time at 37°C. After stopping the enzyme reaction by adding 0.5 ml of 1 M HCl, the DNP–peptide fragments released were extracted by vigorous shaking with 1 ml of ethyl acetate or ethyl acetate–*n*-butanol (1:0.15, v/v) followed by centrifugation at 3000–5000 rpm and room temperature for 10 min to separate two layers completely. The degree of hydrolysis was determined by measuring the absorbance of the organic layer at 365 nm.

Assay for Collagenase Activity

Collagenase activity was determined by measuring the release of radioactivity from ^{14}C-labeled reconstituted guinea pig skin collagen fibrils, as reported previously (2).

RESULTS AND DISCUSSION

DNP–peptides listed in Table 1 were subjected to enzyme digestion by purified tadpole collagenase and human serum peptidase (Table 2). The peptide most susceptible to the action of collagenase was found to be Peptide I. The only site of cleavage by the enzyme was determined to be the Gly–Ile bond, by thin-layer chromatography using a solvent system of

TABLE 1

ANALYTICAL DATA AND PHYSICAL CONSTANTS OF DNP PEPTIDES

DNP-peptide	Mp (decomp.)[a] °C	$[\alpha]_D^{20}$[b]	Molecular formula		Analysis (%) C	H	N
I	215.5–220	−180.4° (c 0.5, DMF)[c]	$C_{36}H_{57}O_{11}N_{13}$ AcOH $1.5H_2O$	Calcd.	48.81	6.90	19.47
				Found	48.86	6.96	19.62
II	194–199.5	+12.5° (c 0.5, DMF)	$C_{31}H_{50}O_{10}N_{12}$ AcOH $1.5H_2O$	Calcd.	47.30	6.86	20.06
				Found	47.42	6.80	19.78
III	230–233.5	−233.2° (c 0.6, 50% CH_3COOH)	$C_{40}H_{61}O_{15}N_{15}$ AcOH H_2O	Calcd.	47.14	6.31	19.64
				Found	46.90	6.64	19.40
IV	210–217	−10.8° (c 0.3, 50% CH_3COOH)	$C_{35}H_{54}O_{14}N_{14}$ AcOH H_2O	Calcd.	45.67	6.22	20.16
				Found	45.85	6.53	20.00
V	240–247	−324.7° (c 0.5, 50% CH_3COOH)	$C_{35}H_{54}O_{12}N_{14}$ AcOH $3H_2O$	Calcd.	45.48	6.60	20.07
				Found	45.32	6.31	20.68
VI	238–256	−272.3° (c 0.6, 50% CH_3COOH)	$C_{41}H_{64}O_{14}N_{14}$ $4H_2O$	Calcd.	46.94	6.92	18.69
				Found	47.12	6.74	18.80
VII	250–257	−267.0° (c 0.5, 50% CH_3COOH)	$C_{40}H_{62}O_{14}N_{16}$ 2AcOH $1.5H_2O$	Calcd.	46.43	6.48	19.69
				Found	46.33	6.44	19.46

[a] Measured by the capillary method, uncorrected.

[b] Measured on a Perkin–Elmer 144 polarimeter.

[c] *N,N*-dimethylformamide.

TABLE 2

COMPARISON OF THE RELATIVE RATES OF HYDROLYSIS OF SEVEN DNP-PEPTIDES BY PURIFIED TADPOLE COLLAGENASE AND HUMAN SERUM PEPTIDASE

	DNP-peptides	Rate of hydrolysis[a] by Collagenase (OD_{365}/hr)	Collagenase OD/OD Peptide III	Serum peptidase (OD_{365}/hr)	Serum peptidase OD/OD Peptide I
I	DNP-Pro-Leu-Gly-Ile-Ala-Gly-L-Arg-NH_2	0.858	9.2	0.237	1.00
II	DNP-Leu-Gly-Ile-Ala-Gly-L-Arg-NH_2	0.001[b]	0.0	0.172[c]	0.73
III	DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH[c]	0.093	1.0	0.001[d]	0.00
IV	DNP-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH[c]	0.001[b]	0.0	0.001[d]	0.00
V	DNP-Pro-Gln-Gly-Ile-Ala-Gly-L-Arg-NH_2[c]	0.307	3.3	0.199	0.84
VI	DNP-Pro-Leu-Gly-Ile-Ala-Gly-Gln-D-Arg-OH	0.276	3.0	0.001[d]	0.00
VII	DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-L-Arg-NH_2[c]	0.204	2.2	0.090	0.38

[a] The average values of three experiments are shown.

[b] No indication of hydrolysis was observed after 16-hr incubation.

[c] DNP-peptide fragment released by enzyme was extracted by ethyl acetate–*n*-butanol (1:0.15, v/v).

[d] No indication of hydrolysis was observed after 2-hr incubation.

[e] Measured at 345 nm.

toluene–pyridine–2-chloroethanol–0.8 M NH_4OH (10:3:6:6) (4). However, the peptide was also hydrolyzed at a fairly good rate by serum peptidase, employed as a nonspecific peptidase. Therefore, a search for a peptide which is susceptible to collagenase, but not to serum peptidase, was undertaken by modifying residues around the site of cleavage. The results obtained by enzyme digestion of the peptides tested indicated that the following three residues are critical for specific hydrolysis by collagenase, preventing the interference by serum peptidase (Table 2). In a sequence of DNP–Pro–X–Gly–Ile–Ala–Gly–Gln–Arg–OH: (i) The presence of Pro residue at the N-terminus was essential for collagenase cleavage, as observed with Peptides II and IV; (ii) Arg residue at the C-terminus should be D-configuration (compare Peptides III and VII for peptidase), and (iii) the presence of Gln residue adjacent to Arg at the C-terminus depresses the effect of peptidase (compare Peptides V and VII for peptidase); (iv) X-residue can be Leu, Gln, or Ala (1), though that peptide was hydrolyzed fastest with Leu. These results suggested that Peptides III and VI were preferable as specific substrates for collagenase. However, the latter peptide was found to be difficult to handle in terms of solubility, compared to the former. Therefore, the overall conclusion was that Peptide III would be the best substrate for collagenase among the peptides tested, even though the hydrolysis rate was only about 30% of that of Peptide VI. As shown in Fig. 1, collagenase activity assayed using Peptide III was a linear function of incubation time and enzyme concentration.

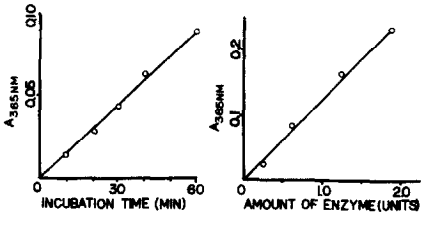

FIG. 1. Kinetic analysis of collagenase activity as functions of incubation time and enzyme concentration at 37°C. (a) Peptide III (0.9 ml of 5×10^{-4} M dissolved in Tris–NaCl–$CaCl_2$ buffer with 0.02% bovine serum albumin was mixed with 0.8 ml of the same buffer with no albumin, then enzyme reaction was started by adding 0.1 ml of purified tadpole collagenase (50 units/ml) at 37°C. Aliquots (0.2 ml) of the reaction mixture were withdrawn at 0, 10, 20, 30, 40, and 60 min to measure the degree of hydrolysis (see Methods). (b) To 0.1 ml each of 5×10^{-4} M Peptide III dissolved in Tris–NaCl–$CaCl_2$ buffer with 0.02% bovine serum albumin was added buffer solution with no albumin to adjust enzyme volume to 0.1 ml at 37°C, then enzyme reaction was started by adding 0.01, 0.025, 0.05, and 0.075 ml of purified tadpole collagenase (25 units/ml), respectively. After 1 hr of incubation at 37°C, 0.5 ml of 1 M HCl was added to stop the reaction and the degree of hydrolysis was determined as described in Methods.

TABLE 3

ASSAY FOR COLLAGENASE ACTIVITY IN SYNOVIAL FLUIDS FROM PATIENTS WITH OSTEOARTHRITIS AND RHEUMATOID ARTHRITIS USING SYNTHETIC SUBSTRATES AND ^{14}C-LABELED RECONSTITUTED GUINEA PIG SKIN COLLAGEN FIBRILS[a]

		Hydrolysis of						Collagenase activity against	
		Peptide III			Peptide IV				
Case	Diagnosis[b]	0 hr	2 hr (OD_{365}/0.1 ml)	Δ	0 hr	2 hr (OD_{345}/0.1 ml)	Δ′	Synthetic substrate[c] (Δ−Δ′/ml·hr)	^{14}C-labeled collagen (units/ml)
S.N.	OA	0.045	0.041	−0.004	0.050	0.041	−0.009	neg[d]	0.00
M.M.	OA	0.042	0.045	0.003	0.038	0.040	0.002	neg[d]	0.00
S.O.	OA	0.045	0.043	−0.002	0.035	0.038	0.003	neg[d]	0.00
M.T.	RA	0.041	0.058	0.017	0.049	0.046	−0.003	0.09[e]	0.00
M.N.	RA	0.045	0.591	0.546	0.045	0.045	0.000	2.73	2.73
T.O.	RA	0.049	0.695	0.646	0.050	0.051	0.001	3.23	2.11

[a] Synovial fluids aspirated from knee joints of the patients were immediately centrifuged at 6000 rpm to remove cell materials and stored at −20°C until used.

[b] OA, osteoarthritis; RA, rheumatoid arthritis.

[c] Activity against Peptide III (collagenase) minus activity against Peptide IV (nonspecific peptidases).

[d] Negligible within an error.

[e] Calculated by only using Δ value, since Δ′ value was negligible within an error.

Peptide III was applied to the assay of collagenase activity in synovial fluids from patients with osteoarthritis and rheumatoid arthritis and the results obtained were compared with those using ^{14}C-labeled collagen fibrils as the substrate (Table 3). The results indicate that Peptide III could be used as a substrate for the assay of collagenase activity in the fluids. A good correlation was observed with the results obtained using ^{14}C-labeled collagen fibril substrate. In this case, Peptide IV, which is not susceptible to the action of collagenase, was used as the reference substrate to monitor the effect of nonspecific peptidases present in the fluids which might affect the results, since collagenase activity could be calculated from the difference in the absorbance of the enzyme digests from both peptide substrates even in the presence of nonspecific peptidase(s) which is active on Peptide III, as suggested from the results obtained with Peptides I and II treated with human peptidase (Table 2).

Application of Peptide III to kinetic analysis of collagenase and to detection of the enzyme in various tissues and body fluids are now in progress.

SUMMARY

Seven kinds of 2,4-dinitrophenyl derivatives of hexa- to octapeptides were prepared in order to find a synthetic substrate for vertebrate collagenase. Analysis of the degree of hydrolysis of the peptides by tadpole collagenase and human serum peptidase(s) demonstrated that 2,4 - dinitrophenyl–L-prolyl–L-glutaminyl-glycyl–L-isoleucyl–L-alanyl-glycyl–L-glutaminyl – D - arginine was the best synthetic substrate for collagenase among the peptides tested. The peptide was successfully applied to the assay of collagenase in synovial fluids from patients with rheumatoid arthritis.

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