SYNTHESIS OF GLYCYL-L-PROLYL-L-LEUCYL-[^{14}C(U)] GLYCYL-L-PROLINE AS A SUBSTRATE FOR COLLAGENASE.

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SUMMARY

The synthesis of the ^{14}C labelled peptide glycyl-L-prolyl-L-leucyl-[^{14}C(U)] glycyl-L-proline (I) with the specific activity of 50 μCi/mMole was performed by the Merrifield solid phase method. The peptide (I) was degraded by Achromobacter iophagus collagenase.

Keys Words : Solid phase synthesis, Collagenase substrate.

INTRODUCTION

The N-protected pentapeptide, N-benzyloxycarbonylglycyl-L-prolyl-L-Leucyl-glycyl-L-proline exhibits substrate properties towards Clostridium histolyticum collagenase which hydrolyses L-leucyl-glycyl linkage of the N-protected peptide (1). In order to investigate the behaviour of the collagenase isolated by Keil and al. (2)(3)(4) from Achromobacter iophagus, we have undertaken the solid phase synthesis of the ^{14}C-labelled pentapeptide : glycyl-L-prolyl-L-leucyl-[^{14}C(U)] glycyl-L-proline (**I**).

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EXPERIMENTAL AND RESULTS.

[^{14}C(U)] glycine, activity of 100 mCi/mMole, 99% radiochemically pure, was from our laboratory. Boc amino acids were from Protein Research Foundation, Japan.

Chloromethylated polystyrene-1% cross-linked divinylbenzene resin (Labs System ; USA) had 1.09 meq Cl/g of the resin. Other reagents and solvents were of analytical grade. The synthesis was performed on an automatic synthesizer Beckman 990 B (USA). The purity of the product was controlled by TLC on Kieselgel 60 (Merck) silica gel plates and the coumpounds were detected by radiochromatogram scanning, ninhydrin and chlore-o-tolidine (at the 100 µg scale). LPLC was performed on Lobar type B (Merck) silica gel columns under pressure (0.5-2 bar) of nitrogen. HPLC was realized on the Waters Associates chromatograph (USA) equipped with U.V. detector Schoeffel (type SF 770). Analytical C_{18} µ-Bondapak column (Waters Associates) and preparative ODS-2 Magnum 9 column (Whatman) were used. Chromatographic solvent systems (all the proportions are given by volumes) were :

A. hexane:ethyl acetate-acetic acid.(20:10:1)

B. acetonitrile:ethyl acetate:acetic acid:water.(2:1:0.5:0.5)

C. n-butanol:acetic acid:water.(4:1:1)

D. acetonitrile:water.(20:80)

The nomenclature is in accord with the IUPAC-IUB rules on Biochemical Nomenclature Biochem.J. 126,773(1972). All optically amino acid are of the L-configuration. The abbreviations are: BOC,t-butyloxycarbonyl;TFA,trifluoroacetic acid;DCC,dicyclohexylcarbodiimide;HOSu,N-hydroxysuccinimide;DMF,dimethylformamide;TLC, thin layer chromatography;LPLC,low pressure chromatography;HPLC, high pressure liquid chromatography.

Boc-[^{14}C(U)]glycine:1 mCi [^{14}C(U)] glycine was used for the preparation of Boc-[^{14}C(U)] glycine according to Schnabel (5). The labelled amino acid was diluted with 1500 mg of unlabelled glycine. The product was compared with an authentic sample of Boc-glycine.(3200 mg;80% yield;Rf A:0.40).

Glycyl-L-prolyl-L-leucyl-[^{14}C(U)] glycyl-L-prolyl-resin :

Boc-L-Proline was esterified to the resin according to Gisin (6) with a substitution level of 0,70 meq L-proline/g polymer as shown by the amino acid analysis. An individual cycle for the incorporation of an amino acid residue was performed according to the scheme summarized in the Table 1 :

TABLE 1.

Step	Reagent, Purpose.	Time (min.)
1	CH_2Cl_2,wash (5x)	1
2	TFA, prewash (1x)	2
3	TFA,deblock (1x)	20
4	CH_2Cl_2,wash (5x)	1
5	10% Et_3N,neutralization (3x)	5
6	CH_2Cl_2,wash (5x)	1
7	Boc-amino acid-CH_2Cl_2 (1x)	10
8	DCC-CH_2Cl_2,coupling (1x)	360
9	CH_2Cl_2,wash	1
10	EtOH,wash (5x)	1

For the DCC coupling with 5 g resin, 3 equivalents of Boc-amino acid and 3 equivalents of DCC in dichloromethane (50 ml) were used. In the case of Boc-leucine, N-methylmorpholine was added for minimizing the diketopiperazine formation (7). For DCC cou-

pling of Boc-L-leucine and Boc-L-proline, an equivalent of HOSu in DMF (15 ml) was added for minimizing the racemization as suggested by Goodman and al. (8). The resin was then checked for complete acylation by ninhydrin (9) and if warranted the coupling was repeated.

Glycyl-L-prolyl-L-leucyl-$[^{14}C(U)]$ glycyl-L-proline.

The peptide was cleaved from the resin (5 g) by treatment with TFA:CH_2Cl_2;80:20(50 ml) (10) and it was liberated from hydrobromide salt by the treatment with acetate form of Amberlite IR-45 ion-exchanger (20 ml), the elution with water (100 ml) followed by 10% acetic acid (50 ml).

The lyophilized material (500 mg) was then chromatographed on silica gel column ; the elution with solvent B gave 250 mg of white powder, which was then rechromatographed on silica gel column with solvent C yielding 150 mg of the product still containing about 10% of $[^{14}C(U)]$ glycyl-L-proline. Therefore the peptidic material was further purified by HPLC using solvent D. In a single run 50 mg of the product was applied to the column yielding the pentapeptide (I) (35 mg) ; overall yield : 7%. Amino acid analysis : Gly 1.90 ; Pro 1.90 ; Leu 1.04 ; RF 0.35 (B) ; 0.25 (C) ;autoradiography : fig. 1. Specific activity : 50 μCi/mMole.

Fig. 1 - Autoradiography of the thin layer chromatography of (I) on silica gel (100 μg of peptide ; solvent C).

The purified peptide was then checked by HPLC in analytical conditions. A shoulder on the peptide peak was observed (fig. 2) being due possibily to the racemization effects or to conformational effects (cis-trans isomerism of the proline) (11).

Racemization test of (I) :

The analysis of the optical purity of (I) was conducted using the Crotalus adamanteus L-amino acid oxydase procedure (12). The sample (1 mg) was hydrolyzed at 110° for 24 hours with azeotropic hydrochloric acid in a sealed tube. The hydrolysate was evaporated to dryness, the residue dissolved in water (0.2 ml) ; the pH was adjusted to 7.2 with Tris-HCl buffer, L-amino acid oxydase (0.25 units) was added. The solution was incubated at 37° for 24 h. More L-amino acid oxydase (0.25 units) was added and the incubation was continued for 48 h. at the same temperature. The solution was diluted with water (1 ml), centrifuged and amino acid analysis was performed on an aliquot : Gly:Pro:Leu:Gly:Pro: 1.09: 1.00: 0,00: 1.09: 1.00. L-proline is completely resistant to L-amino acid oxydase (13).

Collagenase degradation of (I) :

Glycyl-L-prolyl-L-leucyl-[^{14}C(U)] glycyl-L-proline was submitted to the enzymatic attack by Achromobacter lophagus collagenase. Experimental conditions used were as described by Svensson and al. (14). A 10 mM solution (I) in 0.02 M barbital sodium-sodium acetate-HCl, pH 8.5 , was incubated with the enzyme (100 µg/ml) at 37°C. The remaining pentapeptide (I) and the resulting [^{14}C(U)] glycyl-L-proline was isolated from TLC (solvent C) plates and the radioactivity was measured by liquid scintillation technique. The resulting kinetic dependence is shown in fig. 3.

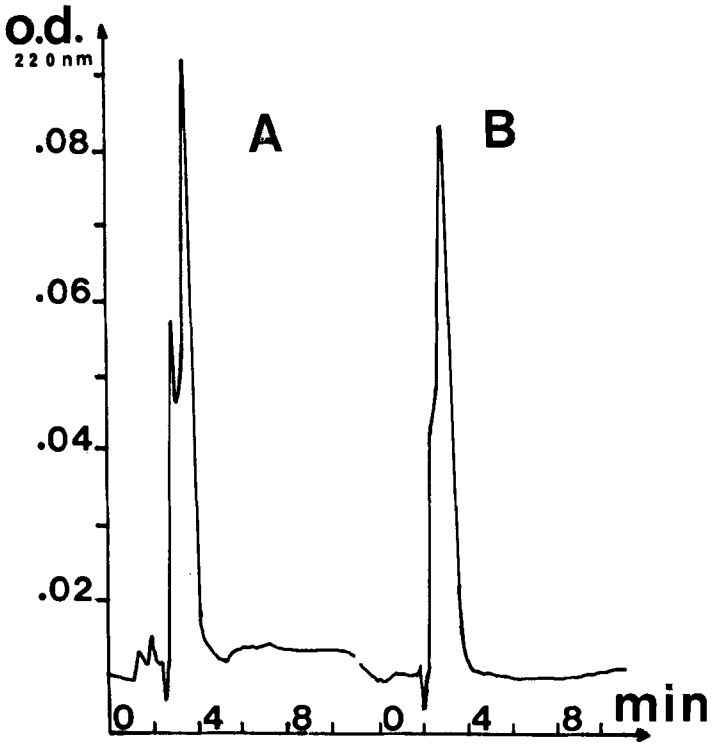

Fig. 2 - HPLC of (I) on analytical C_{18}-μ-Bondapak column (Waters Associates) : 10 μg (I) ; isocratic elution with solvent D, 2 ml/min. A - Before preparative HPLC purification. B - After.

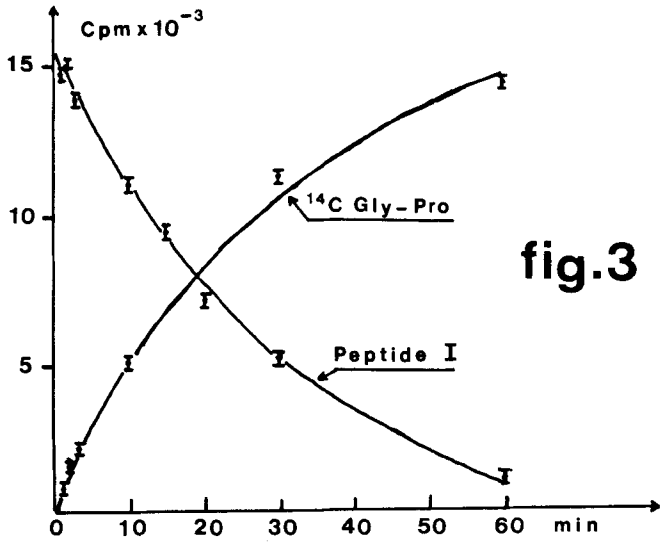

fig.3

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