Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure: Synthesis and Use of the Polymers Poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine Methyl Ester and Poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine Methyl Ester

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Abstract □ The syntheses of the polypeptides poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine methyl ester and poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine methyl ester are described. Using these materials, it was found by cross-reaction and absorption studies that the antibodies to the antigen poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester show specificities for the phenolic hydroxyl group of the tyrosyl residue and the γ-carboxyl group of the glutamyl residue.

Keyphrases □ Polypeptides, linear—synthesis of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester, immunochemical properties □ Antibodies, polypeptide produced—specificity, synthesis and testing of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester □ Immunochemical properties—synthesis and testing of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester □ Antigenic polypeptides, linear—synthesis and use of poly(L-phenylalanyl-L-glutamyl [and L-aspartyl]-L-valylglycyl)glycine methyl ester

It was previously reported that antibodies produced by rabbits against the polypeptide poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) (1–3) are most probably dependent upon the conformation of the antigen (4–7). By the use of the polypeptides poly(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine (II) (8), poly(L-tyrosyl-L-aspartyl-L-alanylglycyl)glycine (III) (9), and poly(L-tyrosyl-L-glutamyl-L-valylglycyl)-glycine (IV) (10), it was shown that these rabbit antibodies possess specificities for the phenolic hydroxyl group of the tyrosyl residue (8) and the γ-carboxyl group of the glutamyl residue (9). All these specificities were deduced by the use of polymers in which only one amino acid residue was modified at a time. It was, therefore, of interest to investigate the ability of these antibodies to cross-react with polypeptides possessing two or more modified residues. For this purpose, the following polymers were prepared and used: poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine methyl ester (V) and poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine methyl ester (VI).

Table I—Amounts[a] of Protein Nitrogen Precipitated by Homologous and Heterologous Polypeptides

Polypeptide	Protein Nitrogen Precipitated at Equivalence Point, mcg.	Protein Nitrogen Precipitated by I after Absorption, mcg.	Protein Nitrogen Precipitated by Polypeptide, %
(Tyr-Glu-Ala-Gly)$_n$Gly (I)	106	0	100
(Phe-Glu-Ala-Gly)$_n$Gly (II)	61	45	57
(Tyr-Asp-Ala-Gly)$_n$Gly (III)	26	78	25
(Tyr-Glu-Val-Gly)$_n$Gly (IV)	106	0	100
(Phe-Glu-Val-Gly)$_n$Gly (V)	63	43	60
(Phe-Asp-Val-Gly)$_n$Gly (VI)	11	94	10

[a] Per milliliter of anti-poly(Tyr-Glu-Ala-Gly)$_n$Gly-1-^{14}C ethyl ester serum.

DISCUSSION

The syntheses of both polymers, V and VI, were performed as detailed in the *Experimental* section. The respective polymerization units were activated by use of the pentachlorophenyl ester (11). The high dilution procedure of polymerization was employed in both cases, since this method has been shown to produce linear high molecular weight polypeptides (1, 2, 4–10). Each polymer was purified by extensive dialysis and then fractionated by successive diafiltrations through membranes[1] into four different molecular weight fractions: $>5 \times 10^4$, $2\text{–}5 \times 10^4$, $1\text{–}2 \times 10^4$, and $<1 \times 10^4$.

Eight rabbits were immunized against I of molecular weight >50,000, using the previously reported protocol (3). It was found that each serum gave a positive precipitin reaction with the homologous polymer I. The serum from each animal was pooled since it was assumed that each rabbit had responded to the same antigenic determinants in this time interval (12). Using the polypeptides V and VI, each of molecular weight >50,000, incremental amounts of these polymers were added to 1-ml. aliquots of this pooled antiserum; cross-reactions occurred with both polymers. However, neither heterologous polymer precipitated as much antibody as the homologous polypeptide I. To quantitate the amounts of antibody not precipitated by either heterologous polymer, two separate experiments were performed. Quantities equal to the equivalence point amounts of each heterologous polypeptide, V and VI, were reacted with the pooled serums. After removal of the precipitates, 30 mcg. of the homologous polypeptide I was added to the two resulting supernatant liquids. Further precipitation was obtained and quantitated by analysis for nitrogen (Kjeldahl) (Table I).

It has been found that the heterologous polypeptides, V and VI, cross-react with anti-I-serum but precipitate less antibody than the homologous antigen I. It appears from these cross-reactions that the conformations of polymers V and VI are similar to that of the homologous antigen I. A similar situation has been shown to exist for the polypeptides II (8), III (9), and IV (10) (included in Table I for comparative purposes). By using the rationale that the determinants of these heterologous polypeptides are in the same orientation as those of the antigen I, it is suggested that observed differences in the binding abilities of these heterologous polymers are due only to those specifically modified amino acid residues. Comparison of the percentage of protein nitrogen precipitated by polymers I and IV has caused the inference to be made that anti-I-serum does not possess a specificity for the alanyl residue (10). Thus, since the percentages of protein nitrogen precipitated by the polymers II and V are similar, their lower abilities to precipitate with anti-I-serum

[1] Diaflo.

may be due only to their deficiency of phenolic hydroxyl groups. Comparison of the percentage of protein nitrogen precipitated by polymers I and III suggested that antibodies to I have a high affinity for the γ-carboxyl group of the glutamyl residue (9). The small amount of precipitate produced by polymer VI is considered as further evidence that antibodies to I have specificities for the phenolic hydroxyl group of the tyrosyl residue and the γ-carboxyl group of the glutamyl residue.

EXPERIMENTAL[2]

***N*-Benzyloxycarbonyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Methyl Ester (VII)**—To a solution of 2.05 g. (0.005 mole) of γ-*tert*-butyl-L-glutamyl-L-valylglycyl methyl ester hydrochloride (13) and 0.51 g. (0.005 mole) of triethylamine in 200 ml. of methylene chloride was added 2.74 g. (0.005 mole) of *N*-benzyloxycarbonyl-L-phenylalanine pentachlorophenyl ester. The mixture was stirred overnight at room temperature and concentrated; the product was dissolved in ethyl acetate, washed with 1 *N* HCl and water, and then dried (sodium sulfate). Concentration under reduced pressure gave an oil, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent to give the fully blocked tetrapeptide. Crystallization from ethyl acetate–hexane yielded 3.0 g. (88%), m.p. 158–160°; $[\alpha]_D^{24}$ −21.6° (c 1.50 in dimethylformamide).

Anal.—Calc. for $C_{34}H_{46}N_4O_9$: C, 62.36; H, 7.08; N, 8.56. Found: C, 62.51; H, 6.95, N, 8.28.

***N*-Benzyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine (VIII)**—To a solution of 3.0 g. (0.00458 mole) of VII in 200 ml. of methanol was added 4.6 ml. of 1 *N* KOH, and the solution was stirred for 90 min. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. The ethyl acetate solution was dried (sodium sulfate) and concentrated under reduced pressure to give the tetrapeptide free acid. This was crystallized from ethyl acetate–hexane to yield 2.7 g. (95%), m.p. 185–186°; $[\alpha]_D^{24}$ −11.4° (c 1.26 in dimethylformamide).

Anal.—Calc. for $C_{33}H_{44}N_4O_9$: C, 61.84, H, 6.92; N, 8.75. Found: C, 61.63; H, 6.80; N, 8.90.

***N*-Benzyloxycarbonyl-L-phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Pentachlorophenyl Ester (IX)**—To a solution of 2.7 g. (0.00422 mole) of VIII in 200 ml. of methylene chloride were added 1.12 g. (0.00422 mole) of pentachlorophenol and 2.0 g. (0.0047 mole) of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide methotoluene-*p*-sulfonate. The mixture was shaken for 2 days at room temperature and then concentrated under reduced pressure to give a solid. This material was washed with water and crystallized from methanol to yield 1.7 g. (45%), m.p. 223°; $[\alpha]_D^{24}$ −19.7° (c 3.04 in dimethylformamide).

Anal.—Calc. for $C_{39}H_{43}Cl_5N_4O_9$: C, 52.69; H, 4.87; N, 6.30. Found: C, 52.43; H, 4.90; N, 6.24.

L-Phenylalanyl-γ-*tert*-butyl-L-glutamyl-L-valylglycine Pentachlorophenyl Ester Hydrochloride (X)—A suspension of 1.7 g. (0.00191 mole) of the tetrapeptide active ester IX and 0.5 g. of 10% palladium-on-carbon in 200 ml. of methanol was treated with 0.07 g. (0.00192 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated. The residue was crystallized from methanol–ether to give 1.1 g. (73%), m.p. 210–212° dec.; $[\alpha]_D^{24}$ −25° (c 0.60 in dimethylformamide).

Anal.—Calc. for $C_{31}H_{38}Cl_6N_4O_7$: C, 47.03; H, 4.84; N, 7.08. Found: C, 46.83; H, 4.91; N, 7.22.

Poly(L-phenylalanyl-L-glutamyl-L-valylglycyl)glycine Methyl Ester (V)—To a solution of 0.42 g. (0.00415 mole) of triethylamine and 0.5 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide was added a solution of 1.1 g. (0.00139 mole) of the polymerizing unit X in 8.3 ml. of dimethyl sulfoxide. The mixture was shaken for 1 week and then centrifuged to yield the fully protected polymer. This material was washed with three 35-ml. portions of water and three 35-ml. portions of ether and dried to give the blocked polymer. Then this material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min., and concentrated under reduced pressure to yield the crude polypeptide V. This polypeptide was suspended in 15 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and dialyzed against distilled water for a day. The precipitated polypeptide V was collected by centrifugation and then lyophilized to yield 0.13 g. (21%); amino acid ratios of an acid hydrolysate were: $Phe_{1.0}$ $Glu_{1.0}$ $Val_{0.9}$ $Gly_{1.0}$.

***N*-Benzyloxycarbonyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Methyl Ester (XI)**—To a solution of 9.0 g. (0.04 mole) of L-valylglycine methyl ester hydrochloride and 4.1 g. (0.04 mole) of triethylamine in 230 ml. of methylene chloride was added 22.8 g. (0.04 mole) of *N*-benzyloxycarbonyl-β-*tert*-butyl-L-aspartic acid pentachlorophenyl ester. The mixture was stirred overnight at room temperature and concentrated, and the product was dissolved in ethyl acetate, washed with 10% citric acid solution and water, and then dried (sodium sulfate). Concentration under reduced pressure yielded an oil, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent. The tripeptide was obtained and crystallized from ethyl acetate–hexane to give 10.0 g. (50%) of the product, m.p. 128°; $[\alpha]_D^{24}$ −25.4° (c 1.18 in dimethylformamide).

Anal.—Calc. for $C_{24}H_{33}N_3O_8$: C, 58.40; H, 7.14; N, 8.51. Found: C, 58.21; H, 7.23; N, 8.50.

***N*-Benzyloxycarbonyl-L-phenylalanyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Methyl Ester (XII)**—A suspension of 6.5 g. (0.0132 mole) of XI and 0.8 g. of 10% palladium-on-carbon in 200 ml. of methanol was treated with 0.482 g. (0.0132 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was dissolved in 200 ml. of methylene chloride and 1.35 g. (0.0132 mole) of triethylamine, and then 7.2 g. (0.0132 mole) of *N*-benzyloxycarbonyl-L-phenylalanine pentachlorophenyl ester was added. The reaction mixture was stirred overnight at room temperature and concentrated under reduced pressure. The product was dissolved in ethyl acetate, washed with 1 *N* HCl and water, and then dried (sodium sulfate). Concentration under reduced pressure yielded a solid, which was chromatographed on a column[3] using chloroform–ethyl acetate (1:1) as eluent to give the fully blocked tetrapeptide. Crystallization from ethyl acetate–hexane yielded 6.5 g. (76%), m.p. 183°; $[\alpha]_D^{24}$ −30.3° (c 3.80 in dimethylformamide).

Anal.—Calc. for $C_{33}H_{44}N_4O_9$: C, 61.84; H, 6.92; N, 8.74. Found: C, 61.73; H, 7.01; N, 8.82.

***N*-Benzyloxycarbonyl-L-phenylalanyl-β-*tert*-butyl-L-aspartyl-L-valylglycine Pentachlorophenyl Ester (XIII)**—To a solution of 6.0 g. (0.00936 mole) of XII in 250 ml. of methanol was added 9.36 ml. of 1 *N* KOH, and the solution was stirred for 90 min. at room temperature and then concentrated under reduced pressure. The residue was flooded with water, acidified with 10% citric acid solution, and extracted into ethyl acetate. The ethyl acetate solution was dried (sodium sulfate) and concentrated under reduced pressure to give 5.9 g. (100%) of the tetrapeptide free acid. This material was dissolved in 250 ml. of methylene chloride, and then 2.49 g. (0.00936 mole) of pentachlorophenol and 4.4 g. (0.0135 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methotoluene-*p*-sulfonate were added. The mixture was shaken for 3 days at room temperature and then concentrated under reduced pressure to give a solid. This solid was washed with water and crystallized from methanol to yield 3.5 g. (43%) of the product, m.p. 164–166°; $[\alpha]_D^{24}$ −22.2° (c 2.48 in dimethylformamide).

Anal.—Calc. for $C_{38}H_{41}Cl_5N_4O_9$: C, 52.17; H, 4.72; N, 6.41. Found: C, 52.04; H, 4.62; N, 6.36.

Poly(L-phenylalanyl-L-aspartyl-L-valylglycyl)glycine Methyl Ester (VI)—A suspension of 3.5 g. (0.004 mole) of the tetrapeptide active ester XIII and 0.5 g. of 10% palladium-on-carbon in 250 ml. of methanol was treated with 0.146 g. (0.004 mole) of dry hydrogen chloride in methanol, and the suspension was hydrogenated for 2 hr. The reacting mixture was filtered, and the filtrate was concentrated under reduced pressure to yield 3.1 g. (100%) of the tetrapeptide hydrochloride. This material was dissolved in 35 ml. of dimethyl sulfoxide and added to a solution of 1.21 g. (0.012 mole) of triethylamine and 0.7 mg. of glycine methyl ester hydrochloride in 5 ml. of dimethyl sulfoxide. The mixture was shaken for a week and then centrifuged to yield the fully protected polymer. This

[2] Melting points were taken with a Mel-Temp apparatus and are uncorrected.

[3] Silicar CC-7.

material was washed with three 35-ml. portions of water and three 35-ml. portions of ether and then dried to give the blocked polymer. This material was treated with 30 ml. of 90% trifluoroacetic acid, stirred for 50 min., and then concentrated under reduced pressure to yield the crude polypeptide VI. This material was suspended in 30 ml. of water and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was dialyzed against distilled water overnight, acidified with 6 *N* HCl to pH 2.5, and then dialyzed against distilled water for a day. The precipitated polypeptide VI was collected by centrifugation and then lyophilized to yield 0.1 g. (6%) of the polymer; amino acid ratios of an acid hydrolysate were: $Phe_{1.0}$-$Asp_{1.0}Val_{1.0}Gly_{1.0}$.

Molecular Weight Determinations—The polypeptide V (0.12 g.) was dissolved in 50 ml. of water by the addition of 1 *N* NaOH to pH 7.4. This solution was separated into four molecular weight ranges by successive diafiltrations through membranes[4]. Each fraction was acidified with 6 *N* HCl, dialyzed, and lyophilized to yield 0.06 g. (mol. wt. >50,000), 0.02 g. (mol. wt. 20,000-50,000), 0.02 g. (mol. wt. 10,000-20,000), and 0.015 g. (mol. wt. <10,000).

A similar process was used to fractionate 0.1 g. of the polypeptide VI, which was comprised of the following: 0.02 g. (mol. wt. >50,000), 0.01 g. (mol. wt. 20,000-50,000), 0.03 g. (mol. wt. 10,000-20,000), and 0.03 g. (mol. wt. <10,000).

Immunochemical Procedures—Eight rabbits were treated with poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I), molecular weight >50,000, at weekly intervals, using the immunization schedule previously described (3); 25 days after the last injection, all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for the precipitin reaction with the homologous antigen I, and serum from each animal gave a positive precipitin reaction. The serum from each animal was pooled, and this combined serum was used for the following experiments. It was assumed that the antibodies produced by each rabbit after the same time interval was directed against the same antigenic determinants of the antigen I.

Quantitative Precipitin Reactions—To 1-ml. aliquots of the pooled rabbit serum were added incremental amounts of the polypeptide I. Each tube was made up to 2 ml. with buffer (0.1 *M* NaCl-0.05 *M* $NaHCO_3$), incubated for 1 hr. at 37°, and then kept at 4° for 48 hr. The tubes were centrifuged in the cold and the precipitates were washed twice with 1 ml. of buffer (0.05 *M* K_2HPO_4-NaOH), pH 7.0. The total amount of protein precipitated was estimated by analysis for nitrogen (Kjeldahl). For V and VI, both of which possessed a molecular weight >50,000, quantitative precipitin reactions were performed using the pooled rabbit serum. These reactions were identical to and run simultaneously with those used for the polypeptide I (Table I).

Absorption Studies—The pooled rabbit serum was treated with the equivalence point amount of the heterologous polypeptides V and VI, each possessing a molecular weight >50,000. The corresponding precipitates were centrifuged out, and the supernatant liquids were poured off into separate tubes. To each supernatant liquid was added 30 mcg. of the homologous antigen, poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I). The tubes were incubated at 37° for 1 hr. and then stored at 4° for 48 hr. The precipitates were collected by centrifugation and washed twice with 1 ml. of buffer solution (0.05 *M* K_2HPO_4-NaOH), pH 7.0. The amount of protein was estimated by analysis for nitrogen (Kjeldahl) (Table I). Controls in which the serum was first absorbed with the homologous antigen I ascertained that the homologous antigen precipitated all of the antibody, since the supernatant liquid gave no further precipitin reaction when 30 mcg. of I was added.

[4] Diaflo, XM 50, UM 20E, and UM 10, Amicon Corp., Lexington, Mass.

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