Immunochemical Studies on Linear Antigenic Polypeptides with a Known Primary Structure

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Abstract □ The specificity of an antibody to poly(L-phenylalanyl-L-glutamyl-L-alanylglycyl)glycine-1-^{14}C ethyl ester (I) was studied using polypeptides in which the phenylalanyl residue was modified to L-tyrosyl and *O*-methyl-L-tyrosyl. Both of these modified polypeptides fit the receptor sites of the antibody to I as well as the homologous antigen. Furthermore, modification of the phenylalanyl residue to a tyrosyl moiety and of the alanyl residue to a valyl moiety caused little loss of the precipitating ability with the antibody to I.

Keyphrases □ Polypeptides, linear antigenic—immunochemical properties evaluated □ Antibodies, polypeptide produced—specificity □ Antigenic polypeptides—immunochemical properties evaluated

The specificity of an antibody formed in response to an antigen is dependent both on the antigenic determinants present in the antigen and on the genetic capacity of the animal to differentiate between and respond to those determinants. Neither the mechanism of specific antigen recognition nor the nature of its genetic control is well understood. With respect to antibody specificity, we have been studying the antigen poly(L - phenylalanyl - L - glutamyl - L - alanylglycyl)-glycine-1-^{14}C ethyl ester (I) (1, 2). To characterize the specificity of the antisera produced by rabbits against the antigen I, using cross-reaction and absorption studies, the following polymers were used: poly(Tyr-Glu-Ala-Gly)$_n$Gly-1-^{14}C ethyl ester (II) (3–5), poly-(OMe-Tyr-Glu-Ala-Gly)$_n$Gly-1-^{14}C ethyl ester (III) (6), and poly(Tyr-Glu-Val-Gly)$_n$Gly-1-^{14}C ethyl ester (IV) (7).

DISCUSSION

Six rabbits were immunized against I using the previously reported protocol (5). The animals were bled 25 days after the last injection of the antigen I. 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 animal had responded to the same antigenic determinants in this time interval (8).

In separate experiments, incremental amounts of each polypeptide (I, II, III, and IV) were added to 1-ml. aliquots of this pooled antisera. All of the polypeptides cross-reacted with this serum. The precipitates were quantitated by analysis for nitrogen (Kjeldahl), and it was found that polymers I, II, and III gave the same amount of precipitin reaction at their equivalence points. The heterologous polymer IV did not precipitate as much antibody as the homologous polypeptide I. These results are shown in Table I.

To quantitate the amount of antibody not precipitated by these heterologous polypeptides, a separate series of experiments was performed. Quantities equal to the equivalence point amounts of each heterologous polypeptide were reacted with the pooled sera. After removal of the precipitates, 30 mcg. of the homologous polypeptide I was added to the resulting supernatant liquids. No further precipitation was observed in each case.

The results suggest that all of the heterologous polypeptides (II, III, and IV) have the same conformation as the antigen I, since all of these polymers cross-react with anti-I sera. From this it was assumed that the determinants of these heterologous polypeptides are in the same orientation as those of the antigen I. Thus, any observed differences in abilities of the heterologous polypeptides II and III to bind with an antibody to I would be due only to the modifications of the phenylalanyl residue. By using this rationale, it is suggested that the antibody-combining site can accommodate the tyrosyl and *O*-methyltyrosyl residues as equally well as the phenylalanyl residue. In the case of polymer IV, the deficiency of the precipitating ability is considered to reside in its valyl moiety. Presumably the larger steric size of the valyl residues slightly inhibits this heterologous polymer to fit the antibody-combining sites.

Table I—Relative Amounts of Protein Nitrogen Precipitated by Homologous and Heterologous Polypeptides

Polypeptide	Micrograms of Protein Nitrogen Precipitated at Equivalence Point[a]	Micrograms of Protein Nitrogen Precipitated by I after Absorption[a]	Percent of Protein Nitrogen Precipitated by Polypeptide[a]
(Phe-Glu-Ala-Gly)$_n$Gly (I)	65	0	100
(Tyr-Glu-Ala-Gly)$_n$Gly (II)	68	0	100
(OMe-Tyr-Glu-Ala-Gly)$_n$Gly (III)	63	0	100
(Tyr-Glu-Val-Gly)$_n$Gly (IV)	58	0	90

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

EXPERIMENTAL

Immunochemical Procedures—Six rabbits were treated with I at weekly intervals, using the immunization schedule previously described (5). Twenty-five days after the last injection, all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for a precipitin reaction with the homologous antigen I and 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 antibody produced by each rabbit after the same time interval was directed against the same antigenic determinants of I.

Quantitative Precipitin Reactions—To 1-ml. aliquots of the pooled rabbit serum was added incremental amounts of the polypeptide I. Each tube was made up to a total of 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 each of the polypeptides (II, III, and IV), quantitative precipitin reactions were performed using the pooled rabbit serum, which were identical to and run simultaneously with that used for the polypeptide I.

Absorption Studies—The pooled rabbit serum was reacted with quantities equal to the equivalent point amount of the heterologous polypeptides II, III, and IV as described previously. The corresponding precipitates were centrifuged out, and the supernatant liquors were poured off into separate tubes. To each of these supernatant liquors was added 30 mcg. of the homologous antigen I. The tubes were incubated at 37° for 1 hr. and then stood at 4° for 48 hr. No precipitation was observed. Controls in which the serum was

first absorbed with the homologous antigen I showed that the homologous antigen precipitated all of the antibody, since the supernatant liquor gave no further precipitation reaction when 30 mcg. of I was added.

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Chemical Constituents of *Salmalia malabarica* Schott and Endl. Flowers

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Abstract □ *Salmalia malabarica* Schott and Endl. flowers have been shown to contain the β-D-glucoside of β-sitosterol, free β-sitosterol, hentriacontane, hentriacontanol, traces of an essential oil, kaempferol, and quercetin.

Keyphrases □ *Salmalia malabarica*—chemical constituents of flowers □ Medicinal plants—*Salmalia malabarica* flowers, chemical characterization

Salmalia malabarica Schott and Endl. (N. O. Malvaceae) is a tall deciduous tree distributed throughout the hotter parts of India and Ceylon and is known to possess medicinal properties. The crimson-red flowers are highly valued in the treatment of leucorrhoea and hemorrhoids and also are used externally for boils, sores, and itch (1, 2). Since no work concerning this plant has been reported in the literature, except for the structure of the gum (3, 4), it was considered of interest to undertake a detailed chemical investigation of the flowers.

The alcoholic extract of the flowers yielded hentriacontane, hentriacontanol, the β-D-glucoside of β-sitosterol, free β-sitosterol, an essential oil, quercetin, and kaempferol. The essential oil was obtained on steam distillation of the alcoholic extractive. The two flavonols were separated by the method of Elsissi and Saleh (5). The isolated compounds were identified on the basis of their physicochemical properties and comparison with authentic samples.

EXPERIMENTAL[1]

Air-dried coarsely powdered flowers (2 kg.) were exhaustively extracted with alcohol (95%) by cold percolation. The solvent (22 l.) was removed under reduced pressure. The alcoholic extractive (18 g.) was successively extracted with petroleum ether, b.p. 40–60° (3 l.), and benzene (2 l.).

Petroleum Ether Extract—On concentration, a white residue, A (900 mg.), was obtained, which was filtered and washed with petroleum ether and ether.

β-D-Glucoside of β-Sitosterol—Residue A crystallized from methanol (charcoal) as colorless needles (250 mg.), m.p. 294–296°; $[\alpha]_D^{30}$ −37.4° (c, 0.9480, pyridine). The compound gave a positive Liebermann–Burchard (L–B) color reaction, showing a typical sequence of colors (pink–violet–blue–green), and also responded to Molisch's test. The IR spectrum showed characteristic peaks at 3450, 1355, and 1370 cm.$^{-1}$.

Anal.—Calc. for $C_{35}H_{60}O_6$: C, 72.84; H, 10.48. Found: C, 72.44; H, 10.68.

Acetate—The acetate was prepared by heating the glucoside with acetic anhydride in the presence of fused sodium acetate (5 hr., 130°). On crystallization from methanol, it was obtained as glistening plates, m.p. 166–168°; $[\alpha]_D^{30}$ −28.8° (c, 1.2, pyridine), and analyzed for $C_{43}H_{68}O_{10}$. Mixed melting point with a known sample of tetraacetate of the β-D-glucoside of β-sitosterol was undepressed.

Hydrolysis of β-D-Glucoside of β-Sitosterol—The glucoside was refluxed with methanolic hydrochloric acid (7%, 8 hr.). The aglycone was worked up as usual and crystallized from ethanol as colorless needles, m.p. 135–137°; $[\alpha]_D^{28}$ −35° (c, 1.2, $CHCl_3$). It gave positive L–B and Salkowski color reactions for sterol. Mixed melting point with an authentic specimen of β-sitosterol showed no depression.

Anal.—Calc. for $C_{29}H_{50}O$: C, 84.07; H, 12.1 Found: C, 83.7; H, 12.3.

β-Sitosterol Acetate—This was obtained as colorless needles (acetone), m.p. and mixed m.p. 128–129°; $[\alpha]_D^{30}$ −38° (c, 1.0, $CHCl_3$).

The sugar moiety in the hydrolysate was confirmed as glucose by paper partition chromatography and preparation of osazone.

Benzene Extract—The filtrate, after the removal of Residue A, was mixed with the benzene extract and the solvents were removed by distillation. The residue, on steam distillation, gave an essential oil (0.003%), having $n_D^{31°}$ 1.428 and $d^{31°}$ 0.8697. The essential oil was not examined further due to its poor yield. The nonvolatile fraction was chromatographed over Brockmann's alumina column using hexane, benzene, and their mixtures as eluent.

Hentriacontane—This was obtained from the hexane eluate as colorless shining plates (800 mg.), m.p. 67–68°. It was optically inactive, and IR spectra showed the absorption band at 731 cm.$^{-1}$ (alkane). Identity of the compound was confirmed on the basis of a

[1] The species determination was done by Dr. S. R. Gupta, Indian Grassland & Fodder Research Institute, Jhansi, India. The plant was identified on February 4, 1970; Accession Number 1, I.G.F.R.I. Herbarium Forest species 404.