Synthesis and Biological Assays of Peptides from a Tuberculin-Active Protein

JAROSLAV ŠAVRDA

Service de la Tuberculose, Institut Pasteur, 75724 Paris, France

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The octapeptide Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly and the hexadecapeptide Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Gln-Met-Arg-Leu, part of a tuberculin-active intracellular mycobacterial protein, were synthesized. The synthetic peptides were shown to possess tuberculin activity by their ability to elicit a delayed-type allergic reaction in skin tests on Mycobacterium tuberculosis-sensitized guinea pigs. Purified protein derivative, the complex mixture of proteins of unknown composition which are excreted into the culture medium by M. tuberculosis and which is in wide use as a tuberculin-active preparation, was shown to cross-react weakly in the radioimmunoassays with the synthetic octapeptide when the 125I-labeled octapeptide and an anti-octapeptide antiserum were used.

The composition of purified protein derivative (PPD; 10), the widely used tuberculin-active preparation obtained from the products excreted by Mycobacterium tuberculosis, is not known, nor has the exact nature been found of the antigenic determinants responsible for the delayed-type allergic reaction when PPD or other mycobacterial extracts are administered intracutaneously to humans or other animals sensitized with tubercle bacilli.

In an attempt to further the present knowledge of this question, I reported previously (9) the synthesis and biological assays of a heptapeptide (70 to 76; Fig. 1), part of an intracellular tuberculin-active protein isolated by Kuwabara from human tubercle bacilli (5, 6). The synthetic heptapeptide (70 to 76; Fig. 1) was found to be devoid of any residual tuberculin activity, in contradiction to reported results (6), but by a radioimmunoassay procedure, its presence in PPD has been established (9).

The present study describes the synthesis of the octapeptide (61 to 68; Fig. 1) and of the hexadecapeptide (61 to 76; Fig. 1), part of the tuberculin-active protein isolated by Kuwabara (6). It is shown that the hexadecapeptide elicits a tuberculin-type reaction in guinea pigs sensitized with human tubercle bacilli.

MATERIALS AND METHODS

Abbreviations. BOC, t-butyl oxy carbonyl; Z, benzyl oxy carbonyl; Me, methyl; Bzl, benzyl; Bu, t-butyl; Tos, p-toluenesulfonyl; TFAOH, trifluoroacetic acid; V, elution volume.

Materials. A M. bovis BCG-Pasteur extract, representing Kuwabara's purification stage 3 (5), was prepared and generously given by M. Gheorghiu, Unité du BCG, Institut Pasteur, Paris, France. Its estimated protein content was 6.250 μg/ml. A M. tuberculosis Aoyama/B extract, representing Kuwabara's purification stage 3 (5), was prepared by L. Meyer in our laboratory. Its estimated protein content was 450 μg/ml, and its measured tuberculin activity was 981 U/mg of protein. N. Carboxypeptidase B (145 U/mg) was purchased from Worthington Diagnostics, Freehold, N.J.; the Bolton-Hunter reagent, 125I-labeled 3-(4-hydroxyphenyl)propionic acid N-succinimidyl ester, was purchased from Amersham Ltd., Amersham, England; Sephade G-15 and G-25 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; and Cellex-D, as well as Bio-Gel P-2 (200 to 400 mesh), was from Bio-Rad Laboratories, Richmond, Calif. All other reagents and L-amino acid derivatives were purchased or generously given as described previously (9).

Experimental procedures for the synthesis of the octapeptide Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly. (i) Silica gel thin-layer chromatography. Silica gel thin-layer chromatography was run in: A, n-butanol–acetic acid–water (3:1:1); B, chloroform–methanol (20:1); and C, chloroform–methanol (2:1). Free amino functions were revealed with ninhydrin, and N-protected peptides were revealed by the chlorine-4,4'-tetramethylidiamino-diphenylmethane procedure (11).

(ii) Z-Glu(BOz)-Gly-OHME (I). A solution of N°-Z-glutamic acid γ-t-buty ester (3.37 g, 10 mmol) and N-methylmorpholine (1.1 ml, 10 mmol) in dry tetrahydrofuran (50 ml) was cooled to −15°C, and ethyl chloroformate (0.96 ml, 10 mmol) was added. After the solution was stirred for 2 min under a calcium chloride guard tube, a cold solution of glycine methyl ester hydrochloride (1.38 g, 11 mmol) and N-methylmorpholine (1.2 ml, 11 mmol) in dimethylformamide (25 ml) was added; the reaction mixture was further stirred at −10°C for 30 min and then at room temperature for 2 h. The reaction mixture was diluted with ethyl acetate and washed with 5% aqueous sodium bicarbonate, 0.2
M potassium hydrogen sulfate, and water. The organic solution was dried over magnesium sulfate and evaporated to dryness to give the product in the form of an oil. Thus prepared, the protected dipeptide (I) moved a single spot on chromatograms ($R_R$, 0.71) and was used without further purification. The yield was 3.76 g (92%).

(iii) Z-Ser(Bu')-Glu(Obu')-Gly-OME (II). The protected dipeptide (I) (4.08 g, 10 mmol) was dissolved in dimethylformamide (20 ml) and submitted to catalytic hydrogenolysis in the presence of 10% palladium on charcoal (Pd-C) (400 mg) and 6 N hydrochloric acid (1.2 ml, 10 mmol). At the end of the reaction, the solution was diluted with methanol and titrated to pH 4.5 with 1 N hydrogen chloride in methanol. The filtered solution was evaporated to dryness, and the residual oil was triturated in ether and left under ether in the refrigerator overnight. After the ether was discarded, the γ-t-buty1-L-glutamyl-glycine methyl ester hydrochloride ($R_R$ for solvent system A [$R_A$], 0.47) was obtained as an oil (3.05 g, 98%) and was coupled to $N^\alpha$-Z-serine β-t-buty1 ester (2.89 g, 9.8 mmol) by the procedure described for the preparation of dipeptide (I). After the usual work-up, the product crystallized out of a concentrated ethyl acetate solution and was washed with ether and dried. The yield was 4.21 g (78%) (mp 114 to 116°C [uncorrected]; $[\alpha]_D^{22} = -13.4 \pm 0.5^\circ$ [concentration] = 2.0% in methanol; $R_R$, 0.62). Analysis: calculated for $C_{27}H_41N_3O_9$: C, 58.79; H, 7.49; N, 7.62. Found: C, 58.73; H, 7.58; N, 7.44.

(iv) Z-Glu(Obu')-Ser(Bu')-Glu(Obu')-Gly-OME (III). The protected tripeptide (II) (5.52 g, 10 mmol) was dissolved in methanol (80 ml) and submitted to catalytic hydrogenolysis in the presence of 10% Pd-C and acetic acid (1 ml). At the end of the reaction, the solution was titrated to pH 4.5 with 1 N hydrogen chloride in methanol, filtered, and evaporated to dryness. The β-t-buty1-L-ser1-γ-t-buty1-L-glutamyl-glycine methyl ester hydrochloride (3.46 g, 9.6 mmol) was obtained as a solid by trituration of the residual oil in ether-n-hexane (1:1) and was coupled to $N^\alpha$-Z-glutamatic acid γ-t-buty1 ester (3.24 g, 9.6 mmol) by the procedure described for the preparation of dipeptide (I). After the usual work-up, the product crystallized out from ethyl acetate upon addition of an equal volume of n-hexane. The yield was 6.51 g (92%) (mp 148 to 150°C [uncorrected]; $[\alpha]_D^{22} = -19.2 \pm 0.5^\circ$ [c = 2.0% in methanol]; $R_A$, 0.89; $R_R$, 0.62). Analysis: calculated for $C_{36}H_56N_4O_{12}$: C, 58.68; H, 7.66; N, 7.60. Found: C, 58.73; H, 7.69; N, 7.55.

(v) Z-Ser(Bu')-Glu(Obu')-Ser(Bu')-Glu(Obu')-Gly-OME (IV). The protected tetrapeptide (III) (3.68 g, 5 mmol) was dissolved in methanol (50 ml) and submitted to catalytic hydrogenolysis in the presence of 10% Pd-C and acetic acid (0.7 ml). At the end of the reaction, the solution was titrated to pH 4.5 with 1 N hydrogen chloride in methanol, filtered, and evaporated to dryness. The γ-t-buty1-L-glutamyl-β-t-buty1-L-ser1yl-γ-t-buty1-L-glutamyl-glycine methyl ester hydrochloride (3.05 g, 4.8 mmol) crystallized out by trituration of the residual oil in ether and was coupled to $N^\alpha$-Z-serine β-t-buty1 ether (1.42 g, 4.8 mmol) by the procedure described for the preparation of dipeptide (I). The thick reaction mixture was diluted with ethyl chloride, washed as usual, and evaporated to dryness. The solid residue was dispersed in ethanol, ether was added, and the crystalline product was filtered and washed with ether. The yield was 3.88 g (92%) (mp 222 to 224°C [uncorrected]; $[\alpha]_D^{22} = -18.5 \pm 0.5^\circ$ [c = 0.5% in methanol]; $R_A$, 0.93; $R_R$, 0.55). Analysis: calculated for $C_{42}H_67N_5O_{14}$: C, 58.69; H, 7.90; N, 7.96. Found: C, 58.71; H, 7.95; N, 8.03.

(vi) Z-Ser(Bu')-Glu(Obu')-Ser(Bu')-Glu(Obu')-Gly-OH (V). The fully protected pentapeptide (IV) (4.40 g, 5 mmol) was dissolved in dioxane (160 ml) and water (16 ml), and the methyl ester was saponified at pH 11.5 by the addition of 1 N sodium hydroxide. At the end of the reaction, the product precipitated out upon the addition of 2 M potassium hydrogen sulfate to pH 2.5 and upon the dilution of the reaction mixture with water. After being washed with water and dried, the product was recrystallized from ethyl acetate. The yield was 3.68 g (85%) (mp 210 to 212°C [uncorrected]; $R_A$, 0.68). Analysis: calculated for $C_{34}H_59N_4O_{12}$: C, 57.06; H, 7.87; N, 7.92. Found: C, 57.19; H, 7.80; N, 7.96.

(vii) H-Ser(Bu')-Glu(Obu')-Ser(Bu')-Glu(Obu')-Gly-OH hydrochloride (VI). The partially protected pentapeptide (V) (4.33 g, 5 mmol) was dissolved in methanol (250 ml) and water (5 ml) and was submitted to catalytic hydrogenolysis in the presence of 10% Pd-C and 1 N hydrochloric acid (5 ml, 5 mmol). The filtered solution was evaporated to a small volume. The repeated addition of ethanol and evaporation gave a gel, which on trituration in n-hexane yielded the product as an amorphous powder. Pentapeptide hydrochloride (VI) moved as a single spot on chromatograms ($R_A$, 0.54) and was used without further purification. The yield was 3.69 g (96%).

(viii) BOc-Asp(Obu')-Gly-OBzI (VII). $N^\alpha$-BOC-aspartic acid γ-t-buty1 ester trifluoroacetate (3.23 g, 9.6 mmol) was obtained as a solid by the trituration of the residual oil in ether and coupled to $N^\alpha$-BOC-aspartic acid β-t-buty1 ester (2.78 g, 9.6 mmol) by the procedure described for the preparation of dipeptide (I). After the usual work-up, the product, which could not be induced to crystallize, was obtained after evaporation of the solvent (ethyl acetate) and drying to a foam. The yield was 4.17 g (88%). Analysis: calculated for $C_{30}H_38N_4O_{12}$: C, 58.40; H, 7.15; N, 8.52. Found: C, 58.20; H, 7.19; N, 8.48 ($R_R$, 0.52).

(ix) BOC-Asp(Obu')-Gly-OH (VIII). The protected tripeptide (VII) (4.93 g, 10 mmol) was dissolved in ethanol (150 ml) and submitted to catalytic hydrogenolysis in the presence of 10% Pd-C. The filtered solution was evaporated to dryness, and the residual oil was dissolved in ether. Upon the addition of

FIG. 1. Part of Kuwabara's protein.
dicyclohexylamine (2.0 ml, 10 mmol), the N\textsuperscript{\(\beta\)}-BOC-\(\beta\)-t-butyI-L-aspartyl-glycyl-glycine dicyclohexylammonium salt crystallized out and was washed with ether. The yield was 5.26 g (90%) (mp 112 to 115°C [uncorrected]). The product was suspended in ethyl acetate and washed with 0.2 M potassium hydrogen sulfate and water. The organic solution was dried over magnesium sulfate and evaporated to dryness. The residual oil was redissolved in ether (20 ml), and \(n\)-hexane was added dropwise to the solution to precipitate the N\textsuperscript{\(\beta\)}-BOC-\(\beta\)-t-butyI-L-aspartyl-glycyl-glycine as a fine powder. The yield was 3.12 g (77%) (mp 78°C after sintering [uncorrected]; \(\text{[\(\alpha\)]D}{^2}_{20} = -4.9 \pm 0.5^\circ\) [c = 2.0% in methanol]; \(R_{FA}\), 0.59; \(R_{GC}\), 0.23). Analysis: calculated for \(C_{12}H_{23}N_3O_8\): C, 50.61; H, 7.24; N, 10.42. Found: C, 50.55; H, 7.62; N, 9.85.

(x) BOC-Asp(OBu')-Gly-Gly-Ser(Bu')-Glut(OBu')-Ser(Bu')-Glu(OBu')-Gly-OH (IX). (VIII) (80 mmol) and \(N\)-hydroxysuccinimide (230 mg or 2 mmol) were dissolved in dioxane (10 ml) and ethyl acetate (2 ml). The solution was cooled to 0°C, and dicyclohexylcarbodiimide (412 mg, 2 mmol) in ethyl acetate (2 ml) was added. The reaction mixture was left to stir at 0°C for 30 min and then at 4°C overnight. After dilution with ethyl acetate (50 ml), the reaction mixture was left in the refrigerator for 2 h, the crystallized dicyclohexyl urea was filtered off, and the solution was evaporated to dryness. Thus prepared, 1-succinimidyl ester of tripeptide (VIII), obtained as an oil (1.00 g, 2 mmol) was dissolved in dimethylformamide (20 ml) and added to a solution of pentapeptide hydrochloride (VI) (1.24 g, 1.62 mmol) in dimethylformamide (35 ml) containing triethylamine (0.90 ml, 6.5 mmol). The reaction mixture was stirred for 24 h at room temperature, the pH was adjusted to 3 with 0.2 M potassium hydrogen sulfate, and the product was precipitated by the addition of water, filtered, and washed with 0.05 M potassium hydrogen sulfate and water. The dry, crude protected octapeptide was extracted with hot acetone, and after an insoluble contaminant was centrifuged off, the clear solution was evaporated to a small volume. In the addition of \(n\)-hexane, the partially protected octapeptide (IX) precipitated out. The yield was 1.48 g (82%) (mp 210°C with decomposition [uncorrected]; \(R_{GC}\), 0.64). Analysis: calculated for \(C_{42}H_{72}N_3O_{10}\): C, 54.84; H, 7.94; N, 10.03. Found: C, 54.36; H, 7.91; N, 9.82. The amino acid analysis of an acid hydrolysate of the product gave Asp, 0.95; Ser, 1.73; Gly, 2.09; Gly, 3.00.

(xi) H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-OH (X). The partially protected octapeptide (IX) (359 mg, 0.5 mmol) was dissolved in TFAOH (20 ml) at 20°C and left to stand for 45 min. Ammonia solution in absolute ethanol (20 ml) was added dropwise to the solution in 20°C under reduced pressure, the trifluoroacetate of the free octapeptide (X) was obtained as a solid by trituration of the residual oil in ether. The yield was 421 mg (99%). The product was dissolved in 25 mM sodium chloride (1.4 ml), 1 N sodium hydroxide (approximately 250 ml) was added to bring the pH of the solution to 3.5, and the product was chromatographed on a column of Bio-Gel P-2 (1.6 by 84 cm) prepared in 25 mM sodium chloride. The chromatographically pure material which emerged at a \(V_s\) of 75 to 85 ml was lyophilized, redissolved in water (1 ml), and desalted on Bio-Gel P-2. The yield of the lyophilized pure octapeptide (X) was 232 mg (63%) (\(R_{FA}\), 0.03). Paper electrophoresis by the procedure described by Offord (7) gave a ninhydrin-positive spot with the expected electrophoretic mobility (\(m = 0.82\)). The amino acid analysis of an acid hydrolysate of the product gave Asp, 0.97; Ser, 1.74; Gly, 2.03; Gly, 3.00.

Experimental procedures for the synthesis of the hexadecapeptide Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Glu-Gln-Met-Arg-Leu (i) BOC-Lys(Z)-Asn-Gly-Ser-Gln-Met-Arg(Tos)-Leu-OBzl (XI). N\textsuperscript{\(\beta\)}-BOC-\(\beta\)-asparaginyl-glycyl-L-seryl-L-glutaminyl-L-methionyl-(\(\omega\)-Tos)-L-arginyll-L-leucine benzyl ester (9) (2.30 g, 2 mmol) was dissolved in TFAOH (60 ml) at 20°C in the presence of thioanisole (11.7 ml, 100 mmol) and left to stand for 1 h. After evaporation to dryness at 20°C under reduced pressure, the N\textsuperscript{\(\beta\)}-deprotected heptapeptide trifluoroacetate (\(R_{FA}\), 0.33) was obtained as a solid (2.28 g, 98%) by thorough trituration of the residual oil in ether and coupled at 20°C for 24 h with N\textsuperscript{\(\beta\)}-BOC-N\textsuperscript{\(\beta\)}-aspartyl-L-lysine 1-succinimidyl ester (4) (1.24 g, 2.6 mmol) in dimethylformamide (50 ml), in the presence of N-methylmorpholine (0.29 ml, 2.6 mmol). The crude octapeptide (XI), which precipitated out of the reaction mixture upon the addition of water, was dried, washed with ether, and digested with hot ethanol. On cooling, the pure product was filtered and washed with absolute ethanol. The yield was 2.12 g (75%) (mp 218 to 220°C [uncorrected]; \(R_{FA}\), 0.60). Analysis: calculated for \(C_{21}H_{35}N_3O_{16}\): C, 54.46; H, 6.71; N, 13.89. Found: C, 54.07; H, 6.82; N, 13.75. (ii) BOC-Asp(OBu')-Gly-Gly-Ser(Bu')-Glut(OBu')-Ser(Bu')-Glu(OBu')-Gly-Lys(Z)-Asn-Gly-Ser-Gln-Met-Arg(Tos)-Leu-OBzl (XII). Octapeptide (XI) (706 mg, 0.5 mmol) was dissolved in TFAOH (40 ml) at 20°C and left to stand for 1 h. After evaporation to dryness at 20°C under reduced pressure, the N\textsuperscript{\(\beta\)}-deprotected octapeptide (XI) trifluoroacetate (696 mg, 0.49 mmol) (\(R_{FA}\), 0.39) was obtained as a solid by trituration of the residual oil in ether and coupled to octapeptide (IX) (547 mg, 0.49 mmol) in dimethylformamide (12 ml) at 0°C in the presence of triethylamine (69 µl, 0.49 mmol), N-hydroxysuccinimide (56 mg, 0.49 mmol), and dicyclohexylcarbodiimide (101 mg, 0.49 mmol) (12). After 2 h at 0°C, the reaction mixture was left to stir at room temperature for 5 days. Upon the addition of water to the thick gel formed, the crude hexadecapeptide (\(R_{FA}\), 0.53), contaminated with dicyclohexyl urea, precipitated out. The yield was 1.21 g. Because of unfavorable solubility properties, the crude protect ed hexadecapeptide (XII) was used without further purification.

(iii) H-Asp-Gly-Gly-Ser-Glu-Ser-Glu-Gly-Lys-Asn-Gly-Ser-Glu-Gln-Met-Arg-Leu (XIII). The crude protected hexadecapeptide (XII) (603 mg, 0.25 mmol) was treated with thioanisole (1.47 ml, 12.5 mmol), and 1 M boronic trifluoroacetate) in TFAOH (8) (25 ml, 25 mmol) was added at 0°C. After 5 min at 0°C, the reaction mixture was stirred at room temperature for 1.5 h and evaporated to dryness at 25°C under reduced pressure. Water (100 ml) was added to the residual oil, the aqueous solution was twice extracted with ethyl acetate, and after titration to pH 4.7 with 1 M sodium hydroxide, the solution was again extracted with ethyl acetate and evaporated to a small volume (10 ml) by azotropic distillation under reduced pressure at 35°C in the presence of ethanol. Part of the boric acid that was formed as a by-product, crystallized out, was filtered off, and washed with water. The combined aqueous filtrates (16 ml) were desalted on a column of
Sephadex G-15, and the salt-free, peptide-containing fractions were lyophilized. The yield was 240 mg (0.145 mmol). Paper electrophoresis at pH 6.5 (7) showed this preparation to be a complex mixture of products, among which the required hexadecapeptide (XIII) could be identified by its Sakaguchi-positive reaction and by its complete disappearance after digestion with trypsin. The crude product (240 mg) was dissolved in 0.01 M phosphate buffer (1.5 ml; pH 7.0) and chromatographed on a column of Sephadex G-25 (fine: 1.6 by 85 cm) in the same solvent. The fractions enriched in hexadecapeptide (XIII) (V, 92 to 104 ml) were lyophilized, dissolved in water (1.5 ml), and desalted on Sephadex G-15. After lyophilization of the peptide-containing, salt-free fractions, the yield of the product was 116 mg (0.07 mmol). This material was chromatographed in two portions of 58 mg dissolved in 1 mM phosphate buffer (2.5 ml; pH 6.6) on a column of Cellex-D (2.6 by 43 cm) prepared in the same solvent. After the product on the column was washed with 1 mM phosphate buffer (400 ml), a linear sodium chloride gradient was applied (830 ml of 1 M sodium phosphate buffer [pH 6.6] against 830 ml of 0.4 M sodium chloride-1 mM phosphate buffer [pH 6.6]). The fractions containing the hexadecapeptide (V, 235 to 309 ml) were lyophilized, desalted on Sephadex G-15, and again lyophilized. The total yield of the pure hexadecapeptide (XIII) was 27 mg (16 μmol, 6.5%). Paper electrophoresis at pH 6.5 (7) gave a single ninhydrin- and Sakaguchi-positive spot with the expected electrophoretic mobility (m = 0.16). The amino acid analysis of an acid hydrolysate of the product gave Arg, 1.00; Asp, 1.96; Glu, 3.03; Gly, 3.92; Leu, 1.01; Lys, 1.02; Met, 0.88; Ser, 2.51. Paper electrophoresis at pH 6.5 of a tryptic digest of the product gave three spots identified as Leu (m = 0.00), Asn-Gly-Ser-Gln-Met-Arg (m = 0.34, Sakaguchi positive), Asp-Gly-Gly-Ser-Glu-Glu-Gly-Lys (m = 0.31).

Preparation of octapeptide (X) conjugate. A bovine serum albumin (BSA)-octapeptide conjugate was prepared from BSA (25 mg) and octapeptide (X) (10 mg) by reaction with glutaraldehyde under the conditions described previously (9). The amount of octapeptide coupled to BSA was 17 mol/mol of BSA.

Immunization procedure. Rabbits were immunized with multisite subcutaneous injections of 2 mg of the BSA-octapeptide conjugate in the presence of muramyldipeptide (2) and Freund incomplete adjuvant as described previously (9).

Preparation of 125I-labeled octapeptide. A 5-μg portion of octapeptide (X) in 10 μl of 0.1 M borate buffer (pH 8.6) was added to 500 μCi of Bolton-Hunter reagent (1) (specific activity, ca. 2,000 Ci/mmol); the reaction mixture was left at 0°C for 40 min and developed by silica gel thin-layer chromatography in solvent system A. The immunoreactive radioactive band (Rf A, 0.55) was eluted with approximately 5 ml of a water-methanol (1:1) mixture. This stock solution of labeled peptide (400,000 cpm/10 μl) was kept at <0°C before use.

Enzymatic digestions. Solutions (1% of octapeptide (X), hexadecapeptide (XIII), or PPD (PPD-neutre Pasteur) were digested with trypsin, and the digests were passed on Sepharose-bound soybean trypsin inhibitor as described previously (9). The resulting solutions were further treated with carboxypeptidase B, with an exopeptidase-to-substrate weight ratio of 1:100, at 25°C for 2 h and then heated for 5 min at 100°C. These stock solutions (800 μg of hydrolyzed substrate per 100 μl) were diluted before use with 0.05 M Tris buffer (pH 7.4), containing 0.15 M sodium chloride, 0.1% sodium azide, and 0.5% BSA.

M. tuberculosis Aoyama/B and M. bovis BCG-Pasteur extracts were likewise digested with trypsin and carboxypeptidase B, except that the concentrations of the substrates (protein) were 0.04 and 0.6%, respectively.

Radioimmunoassay procedure. The radioimmunoassays were performed as described previously (9) with 0.05 M Tris (pH 7.4), containing 0.15 M sodium chloride, 0.1% sodium azide, and 0.5% BSA as buffer and an anti-octapeptide (X) antiserum diluted (1:2,000) with buffer to give 50% bound labeled octapeptide (X) in the absence of an unlabeled standard.

Tuberculin tests. Tests on guinea pigs were carried out by J. Augier of the Tuberculin Unit, Pasteur Institute, Paris. Doses of 200 μg of octapeptide (X) or hexadecapeptide (XIII) in 100 μl of phosphate-buffered saline buffer containing Tween 80 were administered intradermally in the ventral side of guinea pigs sensitized 6 weeks before with heat-killed M. tuberculosis Peurois or M. bovis BCG-Pasteur. The appearance of a delayed-type tuberculin reaction was sought at 24 and 48 h after injection.

RESULTS

Peptide synthesis. The octapeptide (X) Asp-Gly-Gly-Ser-Glu-Ser-Gly and the hexadecapeptide (XIII) Asp-Gly-Gly-Ser-Glu-Ser-Glu-Lys-Asn-Gly-Ser-Gln-Met-Arg-Leu were synthesized as described above by standard procedures of classical solution peptide synthesis and, in particular, under conditions known not to give rise to racemization.

Tuberculin activity of octapeptide (X) and of hexadecapeptide (XIII). When a 200-μg dose of octapeptide (X) in 100 μl of phosphate-buffered saline was administered intradermally to guinea pigs sensitized with BCG, a weak skin reaction (4 mm) of low color intensity could be observed after 24 h. The administration of a 200-μg dose of hexadecapeptide (XIII) to BCG-sensitized guinea pigs elicited after 24 h a slightly stronger skin reaction (8 mm) but still of low color intensity. In BCG-sensitized guinea pigs, the administration of antigens (X) and (XIII) provoked little or no induration, whereas the injection of 10 U (approximately 0.1 μg) of PPD to these animals elicited, on the average, an erythematous area of 14 mm in diameter and an induration of 16 mm. However, the administration of a 200-μg dose of hexadecapeptide (XIII) to M. tuberculosis Peurois-sensitized guinea pigs elicited after 24 h a normal tuberculin-type skin reaction with, on the average, an erythematous area of 11 mm in diameter and an induration of 12 mm. In these animals, 10 U of PPD provoked an erythema of 14 mm and an induration of 16 mm (Fig. 2A). The histological examination of biopsies of the skin showed that the
reactions elicited by PPD (Fig. 2B) and by hexadecapeptide (XIII) (Fig. 2C) were similar, except that the reaction caused by the synthetic peptide contained a higher percentage of polymorphonuclear cells.

**Radioimmunoassays.** An antiserum capable of binding 50% of a 10,000-cpm dose of the \( ^{125}\)I-labeled octapeptide (X) at a 1:2,000 dilution and showing a good affinity for this antigen was selected for the radioimmunoassays. In Fig. 3 are summarized the amounts of unlabeled octapeptide (X) and undigested or trypsin- and carboxypeptidase B-digested hexadecapeptide (XIII), PPD, and Aoyama/B or BCG extracts required to displace half of the label in the radioimmunoassays.
250 pg of octapeptide (X)
Asp — Gly
61 68

250 pg of Tr- and COB-digested octapeptide (X) (control)
Asp — Gly
61 68

500 ng of hexadecapeptide (XIII)
Asp — Gly-Lys-Asp — Met-Arg-Leu
61 68 70 74 76

150 ng of Tr-digested hexadecapeptide (XIII)
Asp — Gly-Lys-Asp — Met-Arg-Leu
61 68 70 74

700 pg of Tr- and COB-digested hexadecapeptide (XIII)
Asp — Gly-Lys-Asp — Met-Arg-Leu
61 68 70 74

150 pg of Tr- and COB-digested PPD

180 pg of Tr- and COB-digested Aoyama/B extract

900 pg of Tr- and COB-digested BCG extract

FIG. 3. Amounts of cold antigens added to radioimmunoassays to achieve 50% inhibition of binding of 125I-labeled octapeptide (X) to its antiserum. The number of the amino acid residues in the synthetic peptide sequences is as in Fig. 1. Abbreviations: Tr, trypsin; COB, carboxypeptidase B.

DISCUSSION

A delayed-type allergic reaction was observed when 200-μg doses of octapeptide (X) or hexadecapeptide (XIII) were administered intradermally to M. bovis BCG-sensitized guinea pigs. However, besides the rather small diameter of the weak erythemas observed, only slight inductions could be detected. On the other hand, when 200-μg doses of hexadecapeptide (XIII) were administered to M. tuberculosis-sensitized guinea pigs, a normal tuberculinit-type allergic reaction could be observed with an increased diameter of the erythematous area and of the normally developed induration, comparable to that obtained with 10 U (approximately 0.1 μg) of PPD.

Thus, hexadecapeptide (XIII) is able to elicit a tuberculinit-type reaction and possibly to distinguish, in skin tests, animals sensitized with different species of mycobacteria. Although the amino acid compositions of the tuberculitin-active proteins isolated by Kuwabara from M. bovis BCG and M. tuberculosis Aoyama/B differ slightly (5), the same pentapeptide sequence (Asn-Gly-Ser-Gln-Met) was found in both proteins (Mitsui Pharmaceuticals Inc., Tokyo, Japan; Austrian patent 325.769/C1, A6IK, 10 November 1975, application 56467/3; Chem. Abstr. 84:162906m, 1976). Thus, the different results of hexadecapeptide (XIII) administration to M. bovis- and M. tuberculosis-sensitized guinea pigs might be due to a particular amino acid sequence situated in the N-terminal portion of this peptide as the sequence (61 to 68, Fig. 1) is specific to M. tuberculosis. This hypothesis is supported by the results obtained in the radioimmunoassays. Only 250 pg of the cold synthetic octapeptide (X) were required to displace half of the label in the radioimmunoassays, using antibodies raised against this peptide (conjugated through its N-terminal α-amine to BSA) and the N°,125I-labeled octapeptide (X). For our purposes, however, the advantage of this good sensitivity of the assays was, to some extent, diminished by the great specificity of the antibodies, which were strongly directed to the C-terminal end of octapeptide (X). Thus, when the C-terminal glycine residue of octapeptide (X) was implicated in a peptide linkage, as in the case of hexadecapeptide (XIII), the observed level of recognition, taking into account the molecular weight of hexadecapeptide (XIII), was only 0.1%, 500 ng of which antigen was required to displace half of the label. When the C-terminal glycine residue of octapeptide (X) was implicated in a peptide linkage with only a single lysine residue, as in the case of the trypsin-digested hexadecapeptide (XIII), the level of recognition was still only 0.4%. Only after further carboxypeptidase B digestion was the level of recognition restored to 80% of that of octapeptide (X); the small loss of potency in cross-reactivity was certainly due to the incomplete digestion of hexadecapeptide (XIII) by the proteases. The very low levels of cross-reactivity between trypsin- and carboxypeptidase B-digested PPD and Aoyama/B or BCG extracts and octapeptide (X) in the radioimmunoassays are, therefore, to some degree, underestimated, as the action of the proteases on these substrates was probably not complete.

The observed cross-reaction of trypsin- and carboxypeptidase B-digested PPD with octapeptide (X) is, however, of the same order of magnitude as the one observed in our previous study (9) when the 125I-labeled heptapeptide (70 to 76; Fig. 1) and an anti-heptapeptide antiserum were used in the radioimmunoassays. The similarity of these results seems, therefore, to indicate that the sequences given by Kuwabara (6) for the heptapeptide (70 to 76; Fig. 1) and for octapeptide (X) (61 to 68; Fig. 1) are correct, as is the structure of the synthetic peptides, since the ratio of these peptides in PPD, even in the form of larger protein fragments, can be expected to be 1:1. These considerations lead us to believe that the heptapeptide (70 to 76; Fig. 1) does not represent an antigenic determinant responsible for the delayed-type allergic tuberculitin reaction, as we previously reported (9), and that the reason for the discrepancy between our results and those reported by Kuwabara does not reside in an incorrect sequence of the heptapeptide. (The pentapeptide [70 to 74; Fig. 1] has been
synthesized independently of us and was found to be devoid of tuberculin activity [personal communication, M. Löw, Chemical Works G. Richter, Budapest, Hungary].) The exceedingly low levels of cross-reactivity observed in the radioimmunoassays with the trypsin- and carboxypeptidase B-digested extracts of Aoyama/B and BCG do not permit much interpretation as the reported amounts of antigen added in the radioimmunoassays (Fig. 3) are only extrapolated values from inhibition curves. However, a definite trend emerges from these results, showing better cross-reactivity in \textit{M. tuberculosis} extracts, whether PPD or Aoyama/B, than in the \textit{M. bovis} BCG extract. This trend might only reflect the different concentrations of the same peptide (61 to 68; Fig. 1) in these extracts, but it might also show that a peptide of a different structure than that of octapeptide (X) is present in the \textit{M. bovis} extract, as is indicated by the tuberculin activity results. If the latter is true, the difference of sequence should reside in the C-terminal portion of octapeptide (X).

We are now investigating the apparent ability of our synthetic peptides to distinguish, in skin tests, animals sensitized with different species of mycobacteria and the possibilities of increasing the low specific tuberculinic activity of these peptides.

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**LITERATURE CITED**


