HYPOGLYCEMIC ACTIVITY OF POLYPEPTIDE-p FROM A PLANT SOURCE[1]

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ABSTRACT.—A hypoglycemic peptide, Polypeptide-p, has been isolated from fruit, seeds, and tissue of *Momordica charantia* Linn (bitter gourd). Amino acid analysis indicates a minimum molecular weight of approximately 11,000 (166 residues). Polypeptide-p is a very effective hypoglycemic agent when administered subcutaneously to gerbils, langurs, and humans.

Insulin used in the treatment of diabetes mellitus has usually been obtained in very low yield from animal pancreas, i.e., one pound of pure insulin per 10,000 animals. Side effects of the animal insulin are well known. Recently, insulin has been synthesized by genetic manipulation in *Escherichia coli*, which is a significant scientific achievement.

A number of indigenous drugs have been tried in the past for the treatment of diabetes mellitus. In the tropical world, fruits of *Momordica charantia* (bitter gourd) have been successfully used by diabetic patients; crude extracts have shown hypoglycaemic activity in rabbits (1–3). Khanna *et al.* (4, 5) were able to isolate an active principle earlier called p-insulin or v-insulin from fruits, seeds, and tissue culture of this plant species (6, 7). When administered subcutaneously to human patients, v-insulin showed a significant blood sugar lowering effect (6).

MATERIALS AND METHODS

TISSUE CULTURE.—Seed coats of *M. charantia* were removed, and the seeds were inoculated on revised Murashige and Skoog's medium (8, 9) supplemented with 1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1% agar. The seeds took 5–6 days to germinate and form seedlings. Organized tissue was established (10) from the whole seedlings and maintained for 12–18 months by frequent subculturings of 6–8 weeks in fresh RT medium. This tissue was harvested after the transfer age of 6 weeks and extracted for its polypeptide-p content.

Fruits, soaked seeds, and tissue samples (100 g each) were crushed separately and then frozen. Each of the frozen samples was dissolved in 10 ml of distilled water, 45 ml of 95% ethanol and 3.6 ml of sulfuric acid (99.5%). The mixture was stirred vigorously (5, 11) for 15–20 min at 25–28° and then homogenized by the addition of 60 ml of distilled water and 250 ml of 95% ethanol separately. After each of the mixtures was filtered, enough ammonium hydroxide (28%) was added to adjust the pH of the filtrate to 3. To each of the filtrates, 1.5 liters of acetone was added till a white flocculent precipitate was formed. These mixtures were kept at 5° for 8–10 hr.

The supernatant from each of the containers was decanted off, and the precipitate was dialyzed in a dialysis membrane (36 DM, Union Carbide Corporation, Chicago, U.S.A.: molecular weight cut-off was 6000); distilled water was used to remove the last traces of salt and other dialyzable impurities until the outside water gave a negative test with barium carbonate. The non-dialyzable fraction was collected, dried and crystallized in a 0.0001% solution of zinc acetate in water (12). The excess zinc was removed by washing with ethylenediamine tetracetic acid (EDTA) solution.

Crystallized material was applied to silica gel G coated and activated glass plates along with a standard sample of bovine insulin. The plates were developed in a solvent mixture of *n*-butanol-acetic acid-water; 12:5:2. When the plates were sprayed with ninhydrin (0.25% in acetone) and heated, a single yellow spot (Rf 0.19), which nearly coincided with that of the standard sample of bovine insulin, was observed.

Disc electrophoresis was carried out (10% SDS Biophore Gel, run in tris buffer, operating pH 6.1; 3% acetic acid in lower cell; 90 V, mA 2.5 per tube; Bromophenol blue tracking dye).

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Samples of the crystallized isolate and bovine insulin were separately prepared with SDS biophore buffer containing dithiothreitol and EDTA, injected, and run for 7 hr. Gels collected from the tubes were stained (0.05% Coomassie Brilliant Blue R-250 in 7% aqueous acetic acid) and washed with 10% acetic acid.

The isolate (25 mg) from each of the samples was hydrolyzed with 6N HCl at 100° for 24 hr and filtered. The filtrate was dried and the residue taken up in 50% ethanol. Two dimensional *tlc* was carried out (silica gel G; solvent system, first: *n*-butanol-acetic acid-water, 5:1:1; second: phenol saturated with water; 0.25% ninhydrin in acetone as spraying reagent), and seventeen amino acids were resolved. The isolates were also run in an automatic amino acid analyzer separately (table 1).

TABLE 1. Amino acids of Polypeptide-p of *Momordica charantia* analyzed by automatic analyzer.

Amino acid	μ moles/mg[a]	Molecular number
Aspartic acid	0.273	17
Threonine	0.138	8.7
Serine	0.195	12
Glutamic acid	0.305	19
Proline	0.159	10
Glycine	0.225	19
Alanine	0.240	15
Valine	0.174	11
½ Cysteine	0.058	3.6
Methionine	0.031	2
Isoleucine	0.116	7
Leucine	0.207	13
Tyrosine	0.016	1
Phenylalanine	0.082	5
Histidine	0.066	4
Lysine	0.209	13
Arginine	0.161	10
NH_3	0.431	(27) omit
TOTAL		166 residues
Approximate molecular weight 11000		

[a]Volume used 0.45 ml (0.81 mg).

A derivative of the crystallized material (polypeptide-p-$ZnCl_2$) was prepared in the same manner (12) as bovine insulin (Insulin-$ZnCl_2$). Doses of polypeptide-p and polypeptide-p-$ZnCl_2$ (in 0.9% NaCl as the vehicle) were prepared (1.8 mg/ml equivalent to 40 units) as used in the case of bovine insulin. Immunoassays were also carried out.

PHARMACOLOGICAL TRIALS.—Pharmacological studies involved *Meriones hurrianae* Jerdon (gerbils), males and females, and *Presbytis entillus entillus* Dufrsne (male langurs). 105 gerbils weighing 63±7 g were used in the present work. The animals were divided into groups of five each and all were fasted for 12 hr before the beginning of the experiment. These animals were provided with water *ad libitum*. Polypeptide-p-$ZnCl_2$ (0.5 unit/kg in 0.9% NaCl) was administered subcutaneously. Thirty-five animals were injected with an equal amount of 0.9% saline vehicle. The blood samples were obtained through cardiac puncture, and the total blood sugar was estimated (13) at different time intervals (0, ½, 1, 2, 4, 8, 12 hr). These results were compared with those of the vehicle-treated controls and statistically analyzed (14; table 2).

A total of six healthy adult male langurs of different age groups with large cannines, a well developed pinkish oedematous band, and the sexual skin on the rump were used as experimental non-human primate models. The animals were fed with wheat chapaty (unleavened bread), banana, onion, carrot, potatoes, and soaked Bengal grams and were provided with water *ad libitum*. Continuous veterinary supervision was maintained.

Polypeptide-p-$ZnCl_2$ (0.5 unit/kg in saline) was administered subcutaneously. Fasting blood sugar samples of each of the animals were taken before any dose of drug was given. Blood samples were taken at different time intervals, as shown in table 3. Food was given after 4 hourly blood samples were taken. An equal number of male langurs were kept fasting and injected with saline (0.9% NaCl in water); their blood sugar samples were taken according to the schedule in table 3.

CLINICAL TRIALS.—A total of nineteen patients (15 males and 4 females) suffering from primary idiopathic (15) diabetes mellitus (15–56 hr age group) for a period of three months to eight years were selected for clinical trials. Out of the nineteen patients selected 11 cases were of juvenile diabetes and 8 were of maturity onset diabetes. Diabetic patients suffering from ketoacidosis, cerebrovascular accidents, acute myocardial infarction and renal failure were excluded from this study.

All patients were admitted to medical wards of S.M.S. Hospital, Jaipur, 4–5 days prior to the commencement of the study. Long-lasting insulin was withdrawn from patients 72 hr

TABLE 2. Effect of polypeptide-p-$ZnCl_2$ (0.5 unit/kg)* on the blood sugar levels of fasting *Meriones hurrinae* Jerdon (gerbils) at different time intervals. (Blood sugar mg/100 ml)

Group No.	Treatment	Body wt. (g)	Fasting (12 hr)	½ hr	1 hr	2 hr	4 hr	8 hr	12 hr
1	Vehicle treated controls (35)**	67±5	95±5	93±3[a]	86±9[a]	90±11[a]	89±3[a]	87±5[a]	84±7[a]
	Sugar fall (%)			2.1±0.5	9.5±1.7	5.3±0.7	6.3±0.5	8.4±0.5	11.6±1.2
2	Polypeptide-p-$ZnCl_2$ (70)**	63±7	92±3[3]	71±7[a,1]	60±5[b,1]	36±7[b,2]	44±8[b,2]	47±8[b,2]	57±7[b,1]
	Sugar fall (%)			22.9±2.0	34.8±3.1	60.9±9.3	52.2±1.8	48.9±2.5	38.1±3.7

*1.8 mg/ml = 40 units.

**Figures in parentheses represents the number of gerbils examined (5 animals were used at each time interval in saline vehicle & 10 animals per time interval with polypeptide-p-$ZnCl_2$ treatment).

[1]Significant at 1% level compared with vehicle treated controls.

[2]Highly significant compared with vehicle treated controls.

[3]Non-significant compared with vehicle treated controls.

[a]Significant at 1% level compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.

[b]Highly significant compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.

[a]Non-significant compared with fasting sugar level of vehicle treated controls. All figures are ± S.E.M.

TABLE 3. Effect of polypeptide-p-$ZnCl_2$ (0.5 units/kg)* on the blood sugar level of fasting *Presbytis entellus entellus* Dufresne (Langurs). (Blood sugar mg/100 ml)

Group No.	Treatment	Body wt. (Kg)	Fasting (12 hr)	½ hr	2 hr	4 hr	20 hr	72 hr
1	Vehicle treated controls (3)**	12±3	62±3	62±5	64±7	58±5[a]	63±5	67±3
	Sugar fall (%)			NIL	NIL	6.5±3	NIL	NIL
2	Polypeptide-p-$ZnCl_2$ (3)**	13±5	64±5[3]	53±5[3,a]	29±1.3[1,b]	20±1.1[2,c]	31±7[x,a]	51±5[x,a]
	Sugar fall (%)			17.2±1.9	54.7±2.7	68±1.8	51.6±3.2	20.3±2.6

*1.8 mg/ml = 40 units.
**Figures in parentheses represent the number of langurs examined.
[x]Significant at 5% level compared with vehicle treated controls.
[1]Significant at 1% level compared with vehicle treated controls.
[2]Highly significant compared with vehicle treated controls.
[3]Non-significant compared with vehicle treated controls.
[a]Significant at 5% level compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.
[b]Significant at 1% level compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.
[c]Highly significant compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.
[s]Non-significant compared with fasting sugar level of polypeptide-p-$ZnCl_2$ treated animals.
[s]Non-significant compared with fasting sugar level of vehicle treated animals.
All figures are ± S.E.M.

TABLE 4. Effect of polypeptide-p on blood sugar level in patients with diabetes mellitus.

	No. of subjects	Fasting values 7 A.M. (mean mg%)	Diabetes duration (yrs)	Mean mg% fall in blood sugar level						
				½ hr	1 hr	1.5 hr	4 hr	6 hr	8 hr	12 hr
JUVENILE DIABETES										
Controls	6	305±10.5	4–8	298.3[s] ±3.0	294.4[s] ±3.7	294.3[s] ±3.0	292.5[s] ±3.4	291.6[s] ±3.9	291.9[s] ±4.2	293.1[s] ±4.6
Polypeptide-p	5	304±18.9	4–8	255.4[2,s] ±27.4	210.7[x,s] ±37.4	187.6[x,a] ±41.6	168.7[x,a] ±46.8	176.0[x,a] ±42.9	172.4[1,a] ±40.7	208.6[x,a] ±38.6
MATURITY ONSET DIABETES										
Controls	2	145±5.2	0.3–3.0	140.7[c] ±2.0	140.4[c] ±1.6	139.1[c] ±2.3	138.4[c] ±2.9	138.2[c] ±2.6	137.9[c] ±2.3	138.6[c] ±2.6
Polypeptide-p	6	140.9± 18.6	0.3–3.0	117.5[b,d] ±12.7	111.7[A,d] ±12.0	95.5[b,d] ±19.2	100.7[b,d] ±15.8	101.8[A,d] ±15.8	93.8[b,d] ±19.5	105.0[b,d] ±16.9

Juvenile Diabetes

[x] = Significant at 5% level compared with control.
[1] = Significant at 1% level compared with control.
[2] = Non-significant compared with control.
[s] = Non-significant compared with fasting sugar level of juvenile diabetic controls.
[s] = Non-significant compared with fasting sugar level of juvenile diabetes treated with polypeptide-p.
[a] = Significant at 5% level compared with fasting sugar level of juvenile diabetes treated with polypeptide-p.
All figures are ± S.E.M.

Maturity Onset Diabetes

[A] = Significant at 5% level compared with control.
[b] = Non-significant compared with control.
[c] = Non-significant compared with fasting sugar level of maturity onset diabetic controls.
[d] = Non-significant compared with fasting sugar level of maturity onset diabetes treated with polypeptide-p.

prior to the test, and plain insulin was withdrawn 12–18 hr before the test. Oral hypoglycemics were withdrawn 48 hr preceding the study. A blood sugar sample after the overnight fast was taken at 7 a.m. Polypeptide-p preparation in saline solution was administered subcutaneously in a dose depending on the severity of diabetes mellitus (less than 180 mg/100 ml blood sugar level, 10 units; 180–250 mg/100 ml blood sugar level, 20 units; 250 mg/100 ml of blood sugar or above, 30 units).

After administration of the polypeptide-p preparation, the first three samples were taken at half-hour intervals to record the onset of the hypoglycemic effect. Subsequent samples were taken at different time intervals, as shown in table 4, to show the peak effect and duration of the action of this polypeptide. The blood samples were withdrawn from the medial cubital vein. The subjects were kept fasting during the study; only plain lemon water was given, if desired by the patients. Supervision was maintained for administration of glucose upon development of hypoglycemic symptoms. Blood sugar determinations were performed by the method of Nelson-Somogyi (16).

The control group consisted of eight of the original nineteen patients with diabetes mellitus. Control blood samples were withdrawn at the same time intervals without the polypeptide-p being administered (table 4). Polypeptide-p-$ZnCl_2$ was administered s.c. to three juvenile patients. These patients required smaller doses of this drug than on bovine insulin.

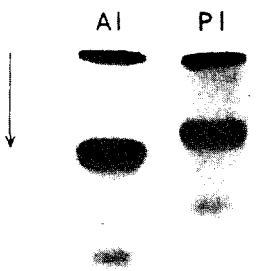

FIG. 1. Polyacrylamide gel electrophoresis pattern of bovine insulin (AI) and plant protein (polypeptide-p; PI) of *M. charantia*.

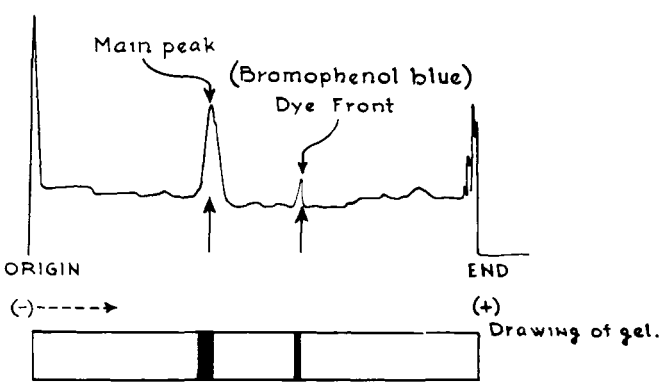

FIG. 2. Scanning of the polyacrylamide gel after electrophoresis on chromoscan (Rf of the main peak 0.41) of plant protein (polypeptide-p) of *M. charantia*.

RESULTS

A single electrophoretic band of dialyzed and crystallized substance (Rf 0.41) was observed which, however, did not coincide (Rf 0.47) with that of bovine insulin (fig. 1). On scanning, a single main peak (Rf 0.41) of pure polypeptide-p was observed (fig. 2).

Two-dimensional tlc and the amino acid analysis (automatic amino acid analyzer) of the polypeptide-p hydrolyzate showed 17 amino acids with a total of 166 residues and a minimum molecular weight of approximately 11000 (table 1). Methionine was the extra amino acid observed in the unknown samples when compared with that of the known bovine insulin. Bio-immunoassays of this polypeptide were found to be negative against bovine insulin.

The pharmacological study revealed that the polypeptide-p-$ZnCl_2$ was long acting in gerbils and langurs and showed a significant blood-sugar-lowering effect (table 2, 3).

Clinical trials showed a hypoglycemic effect of polypeptide-p in juvenile and maturity-onset diabetic patients (table 4). The peak effect in the juvenile diabetic may be between 4–8 hr as compared with 2 hr for crystalline bovine insulin. The peak response in maturity-onset diabetics is not as readily determined as in juvenile diabetics (table 4).

No complaints of any side effects followed administration of polypeptide-p-$ZnCl_2$ to the three juvenile patients. One juvenile patient who expressed frequent heaviness of the head, a swollen face, pain in the stomach, and recurrent episodes of hypoglycemia when kept on crystalline bovine insulin was free of these side effects when maintained continuously on polypeptide-p-$ZnCl_2$ for a period of five months. Immunoassays did not show any cross reaction when tested with bovine insulin.

DISCUSSION

Considering some of the resemblances of polypeptide-p with those of bovine insulin (i.e., extraction procedure, crystallization process, hypoglycemic activity, preparation of polypeptide-p-$ZnCl_2$ (6, 7) and potency), the crystalline isolate has been named p-insulin. However, due to certain differences (i.e. one extra amino acid methionine and negative immunoassays against bovine insulin) the question of a final name for the polypeptide remains open. No apparent side effects were observed when the p-insulin was screened in diabetic patients. Thus, considering its relative hypoglycemic potency and lack of antigencity responses, p-insulin merits additional testing.

Bovine insulin so far is the only remedy against diabetes mellitus. With these new data, a new horizon in the treatment of diabetes mellitus may have been opened. Since the active principle is from a plant source, it is less likely to be antigenic. More clinical trials of action, antigenecity, and various effects of intermediary metabolism in human beings are in progress and shall be reported later.

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