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Note

Gel chromatographic separation of insulin analogues in human serum

KEIJI KAKITA, MASAHARU HORINO, HIDEKI OYAMA, ATSUKO TENKU, SHIGEICHI MATSUMURA and SEIKOH NISHIDA

Division of Endocrinology, Department of Medicine, Kawasaki Medical School, Kurashiki City, Okayama 701-01 (Japan)

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A variety of systems for the identification of insulin molecules by chromatography have been described. Immunoreactive insulin (IRI) in human plasma is separable on Bio-Gel into a major fraction, eluted in the 6000 molecular weight region, corresponding to porcine insulin, and a smaller fraction, eluted in the 9000 molecular weight region, corresponding to porcine proinsulin [1]. Rubenstein et al. [2] fractionated plasma and urine by Sephadex gel filtration and concluded that the insulin-like component recovered in the 9000 molecular weight region was proinsulin. Permutt et al. [3] also reported on the characteristics of high-molecular-weight insulins in insulinoma patients.

Haën et al. [4] reported the existence of abnormalities in the conversion of proinsulin to insulin in normal human plasma. Kimmel and Pollock [5] and Elliot et al. [6] suggested the possibilities of an abnormal insulin in diabetic patients. Two distinct molecules of insulin in rat pancreas were reported by Smith [7, 8].

However, it is very difficult to elucidate the exact mechanism of insulin release and its molecular form in peripheral circulation of the human body, because the specific characterization of the circulating IRI components is limited by the small amount of material present in serum.

This study was undertaken in order to elucidate the heterogeneity of circulating insulin in human serum. Two groups of insulin eluted in the 6000 molecular weight region were fractionated by gel chromatography.

EXPERIMENTAL

Subjects

Four normal young adults (age 19—21 years), four adult onset diabetic patients (age 40—71 years), a borderline case of diabetes mellitus (age 47 years) and three adult onset diabetic patients (age 55—71 years) were studied. There were no significant differences in obesity.

Tests

Fifty-gram oral glucose tolerance tests (O-GTT) were given to all subjects. The serum samples taken 60 min after O-GTT were frozen at —20° until required for use.

Extraction

Extraction of the sera was carried out with minor modifications of the method reported by Oyama et al. [9] within 3 months after sampling. Serum was mixed with water (1:2) and 7.5 ml of a cold mixture of 500 ml of 99.5% ethanol and 10 ml of concentrated hydrochloric acid. The tube was allowed to stand at 4° for 20 h and, after centrifugation at 1900 *g* for 30 min at 4°, the pH of the supernatant was adjusted to 8.3 with ammonia solution. The precipitate was removed by centrifugation at 1900 *g* for 20 min at 4°. After the addition of 0.025 ml of 2 *M* ammonium acetate solution per millilitre of the supernatant, the pH of the solution was readjusted to 5.3 with hydrochloric acid. A 15-ml volume of cold 99.5% ethanol and 50 ml of diethyl ether per 10 ml of the extract were added slowly, and the solution was kept at 4° for 20 h. The precipitate was collected after centrifugation at 600 *g* for 60 min at 4°, dried with nitrogen gas and dissolved in 3 ml of 1 *M* acetic acid.

Gel chromatography

After centrifugation of the above solution, the clear supernatant was applied to a Bio-Gel P-30 column (100—200 mesh, 90 × 1.6 cm) equilibrated with 1 *M* acetic acid and eluted with the same elution buffer at 4°. The column was calibrated with porcine [^{125}I] insulin and porcine [^{125}I] proinsulin. The fraction size was 4.05 ml in eight cases, which included four normal adults and four adult onset diabetic patients, and 2.0 ml in the other four cases, which included a borderline case of diabetes mellitus and three adult onset diabetic patients.

Assay for IRI

After lyophilization, each of the fractions was dissolved in 0.6 ml of 0.1 *M* Tris—hydrochloric acid buffer of pH 7.6 (containing 0.5% of bovine serum albumin) and assayed for IRI. The radioimmunoassay of insulin was performed by the method of Horino et al. [10], utilizing anti-pork insulin guinea pig serum M 8309 and porcine monocomponent insulin (Lot. No. 834098) as standard. Single-component porcine insulin (Lot. No. 615-1082B-108-I) was used as a labelled hormone after iodination with iodine-125 [11].

Dilution tests

The dilution tests were performed with the same insulin assay system to

elucidate the immunogenicity of peaks I and II in the sera from four normal young adults.

Calculations

All values were corrected according to the reported recovery rate of extraction of insulin (0.833) [9] and the calculated recovery rate of gel filtration of labelled porcine insulin (0.647). The results were expressed as mean ± standard error of the mean. All P values were obtained by a paired Student's t-test.

RESULTS AND DISCUSSION

The sera from four normal young adults and four adult onset diabetic patients were analysed with a fraction size of 4.05 ml. The elution profiles of these samples are shown in Fig. 1, where A is from a normal adult and B from an adult onset diabetic patient. The two peaks of insulin (I and II) were well fractionated, and the position of peak II corresponded to porcine [^{125}I]insulin.

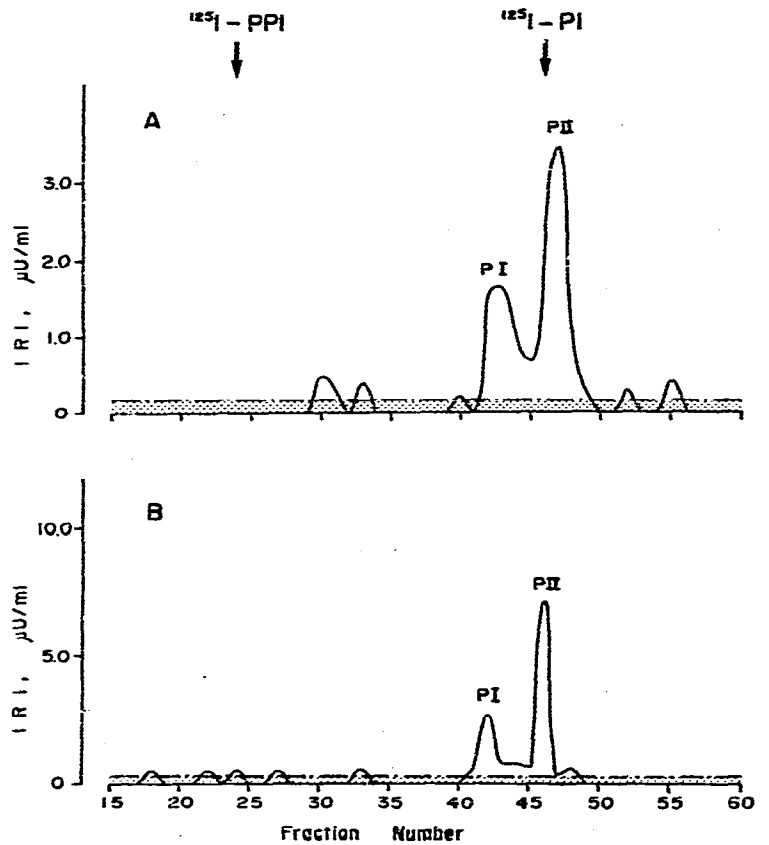

Fig. 1. Elution profiles of extracted human serum insulin on the Bio-Gel column with a 4.05-ml fraction size obtained from a normal and an adult onset diabetic patient. (A) Normal adult (K.T., 20 years old, female); (B) adult onset diabetic patient (K.A., 71 years old, female). Shaded area: detection level of IRI. PPI = porcine proinsulin; PI = porcine insulin.

TABLE I

FRACTIONATION OF INSULIN OBTAINED AT 60 MIN AFTER GLUCOSE LOAD

Results are means ± standard errors of the means. The values were corrected according to the recovery rate of extraction (0.833) and gel filtration (0.647).

Case	Maximum BS level after 50 g O-GTT (mg/dl)	Peaks (p*M*/ml)	
		Peak I	Peak II
Normal ($n = 4$)	126.5 ± 12.4*	0.04 ± 0.02**	0.08 ± 0.03***
Diabetes mellitus ($n = 4$)	267.8 ± 29.2*	0.15 ± 0.02**	0.15 ± 0.04***

*Significant difference ($p < 0.005$).
**Significant difference ($p < 0.01$).
***Significant difference ($p < 0.25$).

From the data in the Table I, the total insulin level (peak I plus peak II) in adult onset diabetic patients (maximum blood sugar level after 50 g of O-GTT: 267.8 ± 29.2 mg/dl) was higher than that in normal adults (maximum blood sugar level after 50 g of O-GTT: 126.5 ± 12.4 mg/dl) at 60 min after 50 g of O-GTT. This result is compatible with those reported by Yalow and Berson [12]. Peak I in adult onset diabetic patients (0.15 ± 0.02 p*M*/ml) was especially increased in comparison with that in normal controls (0.04 ± 0.02 p*M*/ml). However, the structure and function of these compounds are obscure.

In order to confirm the separabilities of these peaks, the sera of the other four adults, namely a borderline case of diabetes mellitus and three adult onset diabetic patients, were analysed by gel chromatography with a fraction size of 2.0 ml. The other conditions for extraction and assay were the same as above. The elution profiles are shown in Fig. 2, where A is from a borderline case of diabetes mellitus and B from an adult onset diabetic patient. In the borderline case, the four prominent peaks were separated. On the other hand, in the three adult onset diabetic patients, the elution profiles of serum insulin were different from that of the borderline case.

Immunoassay of serial dilutions of the insulin peaks I and II in the serum from normal young adults showed immunological identity with porcine monocomponent insulin standard, as shown in Fig. 3. Both fractions should have the same kind of antigenic determinant in their molecules. It has been reported that the anti-pork insulin serum (M 8309) reacts to both insulin and proinsulin, and its immunoreactivity to proinsulin is approximately 66% of that to insulin [13]. However, it is not known which part of the insulin molecule reacts to this antiserum at present. Therefore, the difference between insulin peaks I and II should lie in a different part to the antigenic determinant in the insulin molecule.

The immunoreactivity, the parallelism of immunogenicity among peak I, peak II and porcine monocomponent insulin and the molecular weights of the two fractions suggest that both fractions should be different from somatomedin and non-suppressible insulin-like activity, which have a kind of immunoreactivity of insulin to anti-insulin serum in their molecules. Therefore, the successful fractionation of insulins might be attributed to protein polymorphism.

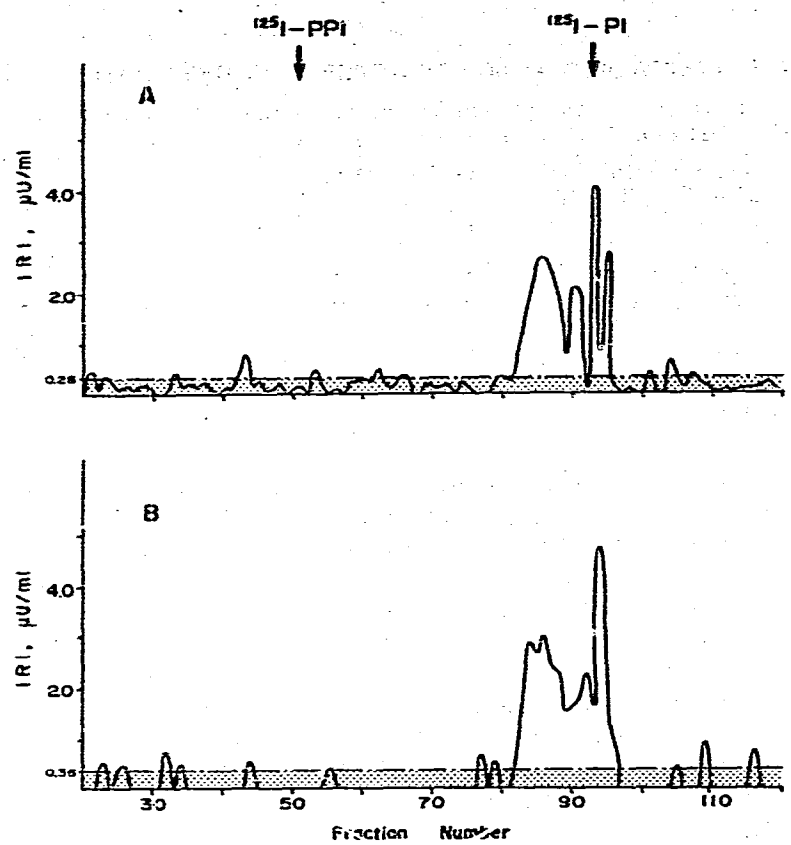

Fig. 2. Elution profiles of extracted human serum insulin on the Bio-Gel column with a 2.0-ml fraction size obtained from a borderline case of diabetes mellitus and an adult onset diabetic patient. (A) Borderline case of diabetes mellitus (F.H., 70 years old, female); (B) adult onset diabetic patient (T.Y., 55 years old, female). Shaded areas: detection level of IRI. PPI = porcine proinsulin; PI = porcine insulin.

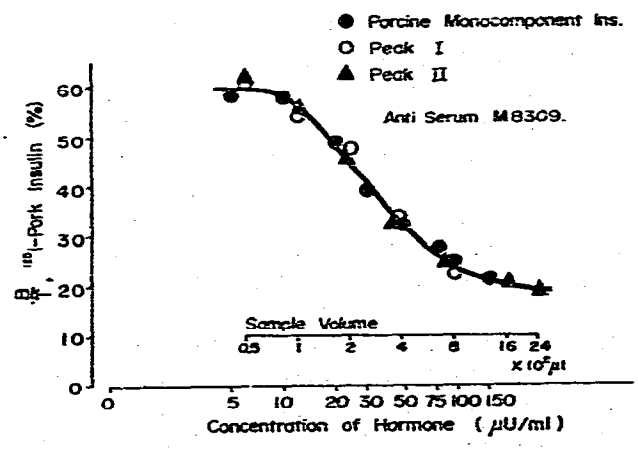

Fig. 3. Comparison of the immunoreactivity of peak I, peak II and porcine insulin in the IRI assay. Anti-pork insulin guinea pig serum (M 8309) was used. Final dilution of antiserum, $1 : 3.2 \times 10^5$. ●, Mean of four determinations; ○, ▲, mean of two determinations.

The following conclusions can be drawn: (1) there are two groups of insulin in human serum, the characteristics of which might be concerned with protein polymorphism; each group could be separable into more than two subgroups; (2) a higher level of insulin peak I is observed in adult onset diabetic patients on diet therapy than that of normal adults at 60 min after 50 g of O-GTT; (3) both insulin analogues (peaks I and II) show the same immunological response with anti-pork insulin serum (M 8309).

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