Immunoreactive proinsulin detected by enzyme-linked immunosorbent assay

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ABSTRACT

A sensitive enzyme-linked immunosorbent assay for human proinsulin was developed by the modification of the method reported using monoclonal antibodies. In the present method, two monoclonal antibodies, an anti-C-peptide antibody bound to microtiter plate, and a biotin-labeled anti-insulin antibody were used. This assay was specific for proinsulin and failed to detect both insulin and C-peptide. The minimal detection limit of this assay was approximately 0.1 pmol/l. Immunoreactive proinsulin levels in serum of normal subjects, ranged from 1.7 to 8.7 pmol/l with the mean of 4.6 pmol/l. The ranges for the intra- and inter-assay coefficients of variance were 3.1–3.7% and 5.0–14.9%, respectively. Reverse phase HPLC analysis of serum of normal subject, as measured with this assay system, revealed two immunoreactive (IR-) forms. One form eluted at the same position as that of authentic proinsulin and the other was detected in a more hydrophilic part of the chromatogram (shorter retention time). Elution profiles of IR-insulin and IR-C-peptide in human serum were also examined by the present reverse phase HPLC and compared to those of IR-proinsulins.

Proinsulin, the biosynthetic precursor of insulin, is synthesized in the β cells of the islet of Langerhans. It is converted to insulin and C-peptide by proteolytic cleavage at amino acid positions 32/33 and 65/66. A direct measurement of immunoreactive (IR-) proinsulin in human serum has encountered difficulty because of cross-reaction with insulin and C-peptide in the conventional proinsulin radioimmunoassay (RIA) (3, 5), and of low proinsulin level in blood.

An elegant method using both insulin antibody and C-peptide antibody based two-site immunoassay was introduced for developing proinsulin assay (4, 7). In the present study, we modified the assay system developed by Kjems *et al.* (7) to establish a reliable enzyme-linked immunosorbent assay (ELISA) for the measurement of serum proinsulin in unextracted specimens. Anti-human C-peptide

monoclonal antibody (Clone No. PEP-001, DAKO A/S, Denmark; 0.5 mg) was dissolved in 0.1 M carbonate buffer, pH 9.0 (100 ml) and 100 μ l was pipetted into each well on the microtiter plate (NUNC, Denmark). The plate was covered with a tape and incubated at 4°C for 3 days and then was washed three times with 350 μ l of 0.05% (w/v) Tween 20 solution (washing solution). The washing solution was aspirated and the plate was stored at 4°C until use. Biotinylated anti-human insulin monoclonal antibody (Clone No. HUI-018, DAKO A/S, Denmark; 1.0 mg) was prepared in the following manner; the monoclonal antibody was dissolved in 0.05 M phosphate buffer pH 7.2 (1.0 ml) and dialyzed against the same buffer solution prior to label. Sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce, U.S.A.) was diluted to 10 mg/ml in phosphate-buffered saline (PBS). Monoclonal antibody solution (500 μ l) and diluted NHS-LC-biotin (460 μ l) were mixed, and the mixture was incubated for 2 h in an ice box. The resulting labelled antibody was applied to a NAP-

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Fig. 1 Standard curve (○) and serial-dilution curves of human serum specimens (●, ◆) containing immunoreactive proinsulin

10 column (Pharmacia Fine Chemical, Sweden). The column was eluted with PBS (1.5 ml) and the purified labelled antibody was diluted in 0.04 M sodium phosphate buffer, pH 7.4 containing 0.1 M NaCl and 0.1% (w/v) Tween 20 (biotin-labelled antibody dilution buffer) and stored at 4°C.

ELISA was designed to produce an immune complex of solid phase anti-human C-peptide monoclonal antibody, standard human proinsulin, biotin-labelled anti-human insulin monoclonal antibody and streptavidin-peroxidase conjugate. Standard diluent buffer was 0.01 M phosphate buffer, pH 7.4, containing 0.06% (v/v) Tween 20, 0.15 M NaCl, 0.1% (w/v) bovine serum albumin and 0.05% (w/v) NaN₃. The assay was carried out in the well on a microtiter plate. Prior to assay, serum was diluted 1:4 with biotin-labelled antibody dilution buffer. In each well, proinsulin standard or diluted serum (100 µl) were mixed. After incubation for 18 h at 4°C, each well was washed 3 times with washing solution and biotin-labelled antibody (100 µl) was added. The mixture was then incubated for 18 h at 4°C and the washing procedure was repeated. Streptavidin-peroxidase conjugate was diluted with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl, 10% (v/v) normal goat serum

and 10% (w/v) polyethylene glycol (PEG) 6000. Diluted streptavidin-peroxidase conjugate (100 µl) was added to each well and the plate was incubated in the dark at room temperature for 1 h. The washing procedure was repeated and 100 µl of enzyme substrate (1,2 phenylenediamine/H₂O₂) were added to each well. After incubation in the dark at room temperature for 0.5 h, the enzymatic reaction was stopped with 100 µl of 1 N H₂SO₄ per well. The color was read with an Immunoreader (model 3550 microplate reader; BioRad, CA, U.S.A.) at 490 nm with reference wavelength of 655 nm.

Fig. 1 shows a typical standard curve and serial-dilution curves of human serum specimens. The detection limit of the assay, estimated from the interpolated response at 2SDs above zero standard was 0.1 pmol/l. Although the affinity of the antibody for insulin (Clone No. HUI-018) used in the present ELISA was approximately 10 times lower than that of Clone No. HUI-001 described by Kjems *et al.* (1, 7), addition of PEG to the streptavidin-peroxidase conjugates dilution buffer resulted in a marked increase in the assay response. In the presence of 10% PEG, the absolute amount of labelled antibody bound to solid phase at proinsulin concentration ranging from 1 to 10 pmol/l was approximately 5-fold greater than that in the absence of PEG.

Dilution of serum containing proinsulin gave displacement curves that were parallel to the standard. The assay range was selected within 0.1–30 pmol/l. In this assay, so called 'high-dose hook' effects which are encountered at high concentration of antigen were not observed up to 1 µmol/l proinsulin. The intra- and inter-assay coefficients of variance (Table 1) were in an acceptable range, with the intra-assay coefficient of variance below 4% and the inter-assay coefficient of variance below 15%. Analytical recovery of proinsulin added to serum in the amount ranging from 0.6–20.0 pmol/l was satisfactory as shown in Table 2. The present ELISA was shown to detect proinsulin in serum specifically as shown in Table 3, while the assay failed to detect both insulin and C-peptide at concentrations up to 1 µmol/l.

The availability of reliable immunoassay for proinsulin has facilitated to define its physiological and pathophysiological significance in patients with β-cell dysfunction. Although elevated serum concentrations of proinsulin have been reported in subjects with impaired glucose tolerance in type 2 diabetics and patients with insulinoma and siblings

Table 1 Assay Precision as Measured with Human Serum Samples

	Proinsulin (pmol/l)		
	Mean	SD	CV (%)
(a) Intraassay variance (n = 10)			
Sample 1	8.4	0.26	3.1
Sample 2	4.1	0.15	3.6
Sample 3	23.1	0.86	3.7
(b) Interassay variance (n = 5)			
Sample 4	6.6	0.79	11.9
Sample 5	26.4	1.31	5.0
Sample 6	11.7	1.74	14.9

SD, standard deviation; CV, coefficient of variance

Table 2 Recovery of Proinsulin in ELISA

Added (pmol/l)	Measured (pmol/l)	Recovery (%)
Sample 1		
0.0	1.3	
0.6	1.9	96
2.0	3.3	98
6.0	7.1	96
20.0	19.9	93
Sample 2		
0.0	6.5	
0.6	7.9	112
2.0	9.0	106
6.0	12.6	101
20.0	23.7	89

Table 3 Cross-Reactivity of Insulin and C-Peptide in ELISA

Peptide	Concentrations (pmol/l)	Cross-reactivity (%)
Insulin	10,000	0
	100,000	0
	1,000,000	0.00015
C-peptide	10,000	0
	100,000	0
	1,000,000	0.00004

of type 1 diabetics (2, 5), physiological functions of proinsulin and proinsulin conversion intermediates are still unclear. In the present study the IR-proinsulin in normal human serum was examined by reverse phase HPLC in combination with the present assay. Serum (40 ml) of normal subjects was diluted with 0.1 N HCl, and the solution was applied to SEP-PAK C₁₈ cartridges which were then washed with 4% (v/v) acetic acid (10 ml), followed by elution with methanol (3 ml). The organic solvent was evaporated to dryness at 30°C and the remaining aqueous solution was lyophilized. The extract was reconstituted with a mixture of 0.01 M ammonium acetate and acetonitrile (65:35 v/v) (1.5 ml), then the solution was subjected to HPLC (TSK Gel 120T, 4.6 × 250 mm) by a modification of the method of Komjati and coworkers (8). The column was eluted at a flow rate of 1.0 ml/min with 0.01 M ammonium acetate pH 4.2, and isocratic elution was carried out with 35% ace-

tonitrile. Fractions (at 1.0 min intervals) were collected, and lyophilized samples were reconstituted with biotin-labelled antibody dilution buffer for assay. As shown in Fig. 2A, the predominant IR-form was eluted in the hydrophilic fractions of the chromatogram with shorter retention times, while other was eluted with the same retention time as that of authentic proinsulin. It has been known that the processing of human proinsulin occurs at the junction of amino acids 32 and 33, and 65 and 66 to yield 32–33 or 65–66 split proinsulins, and 31–32 or 64–65 des-proinsulin (6), respectively. The immunoreactivity detected in the more hydrophilic fractions may result from the presence of proinsulin intermediates. The IR-insulin and IR-C-peptide in the fraction on the above HPLC were examined by both insulin assay kit (Pharmacia & Upjohn) and C-peptide assay kit (Shionogi), respectively. As shown in Fig. 2, B and C, both immunoreactivities, which were eluted with the same retention time as that of authentic insulin or C-peptide, were completely separated from two forms of IR-proinsulin.

The concentrations of intact proinsulin (range

Fig. 2 Elution profile from reverse phase HPLC of IR-proinsulin (A), insulin (B) and C-peptide (C) in human serum as measured with ELISA. Details are described in Materials and Methods. Aliquots from all fractions were lyophilized and assayed with ELISA.

1.7–8.7 pmol/l) measured in the healthy fasting subjects ($n = 46$) are in accordance with results of previous studies (2, 3, 7). The results show clearly that the present assay may effectively be used for the direct measurement of IR-proinsulin in human serum.

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