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Application of size-exclusion chromatography in the investigation of the in vitro stability of proinsulin and its cleaved metabolites in human serum and plasma☆

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ABSTRACT

To help ensure reliability of proinsulin measurements and define the optimal matrix for conducting routine bioanalysis of this prognostic biomarker, we undertook a systematic evaluation of its *in vitro* stability. For this study, we subjected mono-radioiodinated forms of hPI and its cleaved metabolites to size-exclusion chromatography (FPLC™-SEC employing a Superdex-75 10/30 HR column) to characterize their elution profiles following incubation in human serum and plasma. We determined that intact hPI is a substrate for serine-like protease(s) that are present in human serum. Furthermore, RIA analysis of the elution profile of unlabeled peptide demonstrated that the B–C junction is cleaved preferentially. Thus, *in vitro* degradation of hPI represents a potential pathway for the formation of cleaved metabolites. Our findings confirmed that EDTA plasma is the preferred matrix for quantitative determination of intact hPI and its cleaved metabolites. We concluded the SEC strategy employed in this study is broadly applicable to evaluating the *in vitro* stability of other peptides/proteins of diagnostic or therapeutic interest.

1. Introduction

Proinsulin (Fig. 1) and its B-chain-C-peptide cleaved intermediate have gained interest in recent years as potential prognostic biomarkers for pancreatic beta islet cell status [1–3], insulin resistance [4–7], type II diabetes [8–10], increased cardiovascular risk in type II diabetes [11–13], hypoglycemia [14] and pancreatic tumors and cancer [15–17]. Accordingly, much effort has gone into developing improved immunoassay methods for quantitative determination of circulating concentrations of total, intact peptide [3,18–20] and cleaved metabolites [21,22]. Early RIA methodology tended to detect both intact peptide and cleaved metabolites [23–25], while more recent sandwich ELISAs and related immunoanalytical methodologies have permitted the specific detection of intact peptide [3,19,20,21] and cleaved metabolites [22,23,27].

One important aspect for maximizing the reliability of proinsulin measurements concerns its stability in biological matrices. While some studies have suggested that plasma may be superior to serum [3,21,26], detailed information from a systematic investigation of hPI matrix stability is lacking. In this manuscript, I have reviewed our findings concerning the *in vitro* stability of hPI in serum and plasma. For these studies, we subjected mono-radioiodinated forms of hPI to size-exclusion chromatography to characterize the elution profiles following incubation in human serum and plasma. Size-exclusion chromatographic analysis of proinsulin has been reported previously in classical studies of insulin metabolism [28–30] and during the manufacturing process for biosynthetic human insulin [31,32]. In addition to its resolution and speed modern SEC is attractive as a tool for investigating the metabolism of peptides and proteins, because it can be performed under physiological non-denaturing conditions and its semi-preparative nature allows collection of the eluting peaks as fractions for further characterization in terms of their bioactivity

Abbreviations: hPI, human proinsulin; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbant assay; HPLC, high performance liquid chromatography; SEC, size-exclusion chromatography; cpm, counts per min.

☆ This manuscript reviews and summarizes work that was conducted at the Lilly Research Laboratories and has been published previously as poster presentations: (1) R.R. Bowsher, J.D. Wolny, Differential stability of proinsulin in human serum and plasma, Diabetes 42 (Suppl. 1):238A (1993); (2) R.R. Bowsher, P.F. Santa, Serum catalyzes the *in vitro* formation of B-chain-C-peptide junctional split forms of proinsulin, International Diabetes Fed, 15th International Diabetes Fed., Kobe, Japan, 1994.

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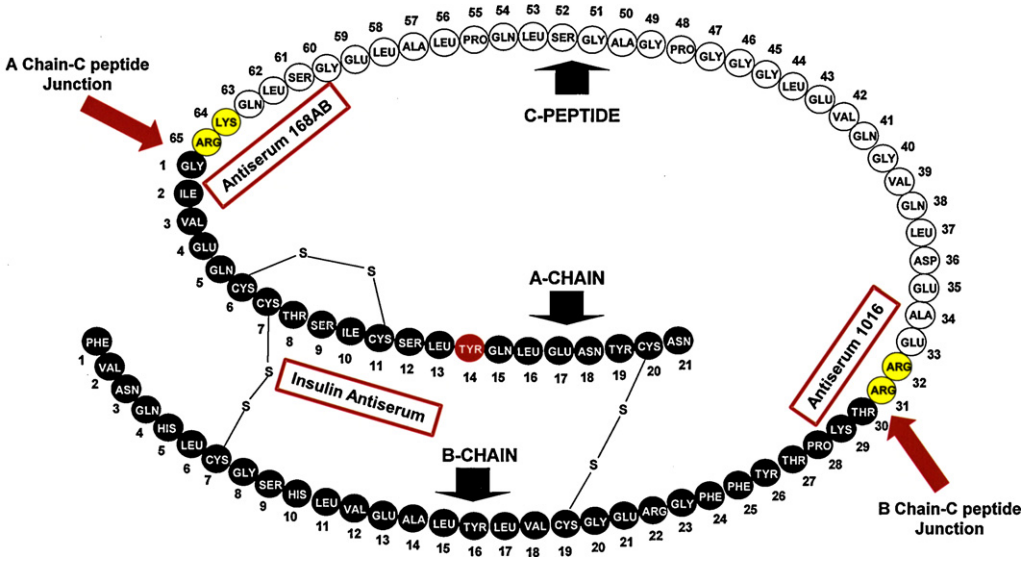

Fig. 1. Primary sequence of human proinsulin. The peptide sequences representing the A and B chains of insulin, as well as the structure of C-peptide are denoted by black arrows. The tyrosine residue that is radiolabeled with [^{125}I] is highlighted in red. See Section 2 for a description of the preparation and C_{18} RP-HPLC purification of the various radiolabeled peptides. Because the radiolabeled Tyr is present in the insulin sequence, the chromatographic profiles of radioactivity denote the elution of insulin-like peptides. The known proteolytic cleavage sites in hPI that comprise the junctions of the A-chain-C-peptide (A–C) and B-chain-C-peptide (B–C) are denoted with arrows. In addition, the dibasic amino acids that represent the protease sensitive residues are highlighted in yellow. The peptide regions that comprise the binding epitopes for the antisera used in this study are indicated by the red boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and immunoreactivity. In this study, SEC analysis permitted sufficient resolution of intact peptide, its cleaved intermediates and insulin to enable detailed investigation of *in vitro* stability. Moreover, this study confirmed that EDTA plasma is a preferred matrix for quantification of intact hPI.

2. Experimental

2.1. Preparation of radiolabeled peptides

Biosynthetic human proinsulin, des(31,32)hPI, des(64,65)hPI and insulin were radioiodinated by a conventional lactoperoxidase method. The mono-[^{125}I]-tyr(A14) peptides were isolated by C18 reversed phase HPLC using isocratic elution with 29% acetonitrile/0.2 M ammonium acetate, pH 5.5 [25,33].

2.2. Blood collection and processing

Samples of antecubital venous blood were obtained from six fasted healthy adult volunteers. Blood samples were allowed to clot at ambient temperature for 30–40 min prior to centrifugation. For plasma, blood was drawn into Vacutainer® tubes containing either K_3 EDTA or Li heparin. After gently mixing the blood and anticoagulant by inverting each tube several times, the plasma was promptly separated by centrifugation at about 3000 × g for 15 min at 4 °C. Samples were combined into pools of serum and plasma, pipetted into aliquots, and stored frozen at −70 °C.

2.3. Proinsulin degradation assay

Each 500-μL reaction was performed in a 12 mm × 75 mm borosilicate glass culture tube and was formed by adding 400 μL of sample (serum, plasma or buffer), 25 μL of 1 M Na HEPES/2% Na azide, pH 7.5, 50 μL of Milli-Q® water and 25 μL of a 10–20 ng/mL solution of radioiodinated hPI or hPI metabolite (approximately 50–100 pM final concentration). Radioiodinated peptides were diluted in 50 mM Na HEPES/0.9% NaCl/0.5% BSA/0.1% Na azide, pH 7.5. For experiments involving protease inhibitors, the 50 μL addition of water was replaced by an inhibitor solution. After incubation in a water bath at 37 °C, the reaction mixture was passed through a 0.45 μ filter and a 200 μL aliquot was analyzed by SEC.

2.4. SEC separation of hPI and related peptides

FPLC™ size-exclusion chromatography was performed using a Superdex-75 10/30 HR column (GE Healthcare). The mobile phase consisted of 50 mM Na HEPES/0.9% NaCl (w/v)/0.1% Na azide (w/v)/0.05% tween-20 (v/v), pH 7.5. The column was eluted at a flow rate of 0.5 mL/min and 0.25 mL fractions were collected in 12 mm × 75 mm tubes. Protein elution was monitored at 280 mn. The elution pattern of radiolabeled peptides was obtained by counting the fractions for 1 min in a gamma counter.

The column void volume, V_o (7.6 mL), was determined with blue dextran, while the total column volume, V_t (19.6 mL) was estimated using a 5% solution of acetone in mobile phase. The elution volume, V_e, of each radiolabeled peptide was expressed in terms of its partition coefficient, K_{av}, and was calculated as follows: $K_{av} = (V_e - V_o)/(V_t - V_o)$. For evaluation of hPI degradation, 40 fractions were collected beginning at 10 mL and ending at 20 mL after each injection. This collection interval comprised matrix components that eluted with K_{av} values between 0.23 and 1.03.

2.5. Degradation of hPI by purified enzymes

Each 500-μL reaction was formed by adding 425 μL of 50 mM Na HEPES/0.9% NaCl/0.1% Na azide/0.5% BSA, pH 7.5, 25 μL of radioiodinated peptide (10–20 ng/mL), and 50 μL of purified enzyme diluted in the HEPES buffer. Following incubation for 1 h in a water bath at 37 °C, a 200-μL aliquot was analyzed by SEC.

2.6. Characterization of hPI metabolites by SEC and RIA

A 2-mL reaction mixture was formed by adding 1.6 mL of serum, 0.1 mL water, 0.1 mL of 1 M Na HEPES/2% Na azide, pH 7.5 and 0.2 mL of hPI (20 nM). After mixing, the reaction was incubated in a water bath at 37 °C. Aliquots (0.5 mL) were obtained at 0, 4 and 24 h. Each aliquot was filtered through a 0.45 μ membrane and a 200 μL was analyzed by FPLC-SEC. Following chromatographic separation,