A Novel HPLC Method for Glutathione-Insulin Transhydrogenase Assay Using Fluorescence Labeled Insulin

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ABSTRACT. The activity of rat liver glutathione-insulin transhydrogenase (GIT) was measured by HPLC. The degradation of fluorescein isothiocyanate-I (FITC-I)-labeled insulin is separated into several peaks, which are bound different amount of FITC-I. We selected mono-fluorescein-thiocarbamylated insulin to estimate the decrease of insulin content and it became possible to assay GIT activity. This novel method was time-saving and simple, and this system could utilize instead of previous method.—**KEY WORDS:** fluorescein-thiocarbamylated insulin, glutathione, glutathione-insulin transhydrogenase, HPLC, insulin.

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Glutathione-insulin transhydrogenase (GIT) is an important enzyme which catalyzes the first reaction of the insulin degradation process. GIT cleaves three disulfide bonds in one molecule of insulin to produce A and B chains [15, 16].

The reduced glutathione (GSH), which acts as a cofactor and substrate of some detoxicating enzymatic reaction eg. glutathione peroxidase or glutathione *S*-transferase, also plays as hydrogen donor for the GIT reaction. It was reported that the insulin level increase in cattle which recieved high energy and protein feeds [1, 2, 9, 10, 21]. Therefore it is considered that the measurement of the activity of GIT may provide information about the metabolism of insulin and GSH in the animal body.

There are few reports on the activities of GIT except for those of Varandani *et al.* who have studied this enzyme for 30 years [16-20]. Only the method developed by Tomizawa *et al.* [14] has been utilized to assay GIT hitherto. In this method, the radioactivity of the trichloroacetic acid-soluble product (A chain) released from substrate, [¹²⁵I]-labeled insulin was measured. However, this method was complicated because it required special facilities and [¹²⁵I] insulin must be handled carefully due to its volatility.

In this paper, we report on a novel method to assay GIT activity by measuring the decrease of the amount of insulin labeled with fluorescein isothiocyanate-I (FITC-I). This method is so easy without any special apparatus and facilities. By this method, GIT research will develop to the studies of a enzymology and metabolism of living organism in future.

MATERIALS AND METHODS

Reagents: Fluorescein-isothiocyanate-I (FITC-I), bovine serum albumin (BSA, Fraction V), 2-vinyl pyridine, acetonitrile were purchased from Wako Pure Chemical Industries, Ltd., and glutathione (reduced form) and bovine insulin from Sigma Chemical Company. General reagents except BSA were of analytical grade.

Animals: Male Fischer rats aged nine weeks and weighing approximately 200 g recieved feed and water *ad libitum*. Prior to killing by bleeding, they were starved over night. The liver was removed after perfusion *in situ* with a 0.9% physical saline solution, and then it was homogenized with four volumes of 0.25 M sucrose. The homogenate was centrifuged at 10,000 ×g for 15 min. The supernatant, postmitochondrial fraction, was used for the enzyme assay.

Preparation of fluorescein-thiocarbamylated insulin: Fluorescein-thiocarbamylated insulin (FTC-insulin) was prepared by the modified method of Maeda *et al.* [11, 12] as follows; 1 mg of bovine insulin and 0.33 mg of FITC-I which is 5 time amount per one insulin molecule was dissolved in 1 ml of 0.5 M carbonate buffer (pH 9.0). The reaction mixture was incubated for 3 hours at 20°C in the dark. FTC-insulin was separated by a Sephadex G-50 column (2.5 × 70 cm). Elution was done with 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl at a flow rate of 21 ml/hr. Each 3 ml eluent was fractionated and monitored at 280 nm. Only one protein peak from the 21th to 59th fraction was

observed but content ratios of mono-FTC-insulin to di-FTC-insulin which were analyzed by HPLC as shown in Fig. 1 were rich in the latter half fractions of the peak. The mono-FTC-insulin-rich fractions (from the 38th to the 42th fraction) were pooled and used as a substrate for GIT enzyme assay. The fraction was freeze-dried and stored at -20°C until use.

Enzyme assay: For dose-dependence study, the reaction mixture (1 ml) contained a different amounts of rat liver postmitochondrial fraction, 1 mM GSH, 1 mg BSA, 2.6 μmole mono-FTC-insulin, 5 mM EDTA and 0.1 M sodium phosphate buffer (pH 7.5). The reaction was started at 37°C by the addition of FTC-insulin and terminated 5 min later by the addition of 10 μl of 2-vinyl pyridine. Then the reaction mixture was filtrated through quartz wool. The clarified sample was applied to HPLC for analysis. And for time course study, a 40 μl aliquot of the rat liver postmitochondrial fraction was incubated with FTC-insulin, GSH and BSA. The decrease of the content of mono-FTC-insulin was observed until 20 min.

Chromatographic procedure: HPLC analysis was carried out by modifying the method of O'Hare and Nice [13]. Briefly, 100 μl of a sample was injected into a Nova Pak C_{18} column (8 mm $\times$ 10 cm, Waters Japan Co. Ltd.) connected to a liquid chromatograph equipped two solvent delivery systems and a programmer, ALC/GPC-244 (Waters Japan Co., Ltd.) and a fluorescence spectrophotometer, 650-10LC (Hitachi Ltd.). The elution was done as described in Fig. 1. The fluorescence intensity was measured at 515 nm by excitation at 489 nm. GIT activity was estimated from the peak height of mono-FTC-insulin by enzyme treatment.

RESULTS

To analyze the activity of GIT as insulin degradation, it is necessary to exactly determine the amount of insulin. With high sensitivity of fluorescence detection, FTC-insulin could be assayed in nanogram order. Fig. 1 showed an example of chromatograms compared an enzymatic treated sample with nonezymatic control by reverse phased HPLC. The two major peaks were identified as mono-FTC-insulin and di-FTC-insulin from a ratio of fluorescence intensity to protein content, respectively. The content of di-FTC-insulin was 27% of that of mono-FTC-insulin, that was 0.71 μmole in 1 ml of

Fig. 1. Reverse-phase HPLC patterns of FTC-insulin with and without GIT. Samples were prepared by incubation insulin substrate with 40 μl of liver enzyme (B) or same volume of buffer as control (A). Details were described in MATERIALS & METHODS. Samples were applied on Nova Pak C_{18} column equilibrate with 20% acetonitrile in 0.1 M $\text{NaH}_2\text{PO}_4\text{-H}_3\text{PO}_4$ buffer (pH 2.1), and eluted with a linear gradient of acetonitrile in same phosphate buffer from 20% to 52% at a flow rate of 2 ml/min for 20 min. Mono-FTC-insulin (I) and di-FTC-insulin (II) were eluted at 15 min and 13 min, respectively.

reaction mixture. Degraded products were not detected but the decrease of fluorescence intensity in both peak was observed. For simplification to estimate the amount of degraded insulin, we observed the reduction of mono-FTC-insulin peak. This is the reason why we used a mono-FTC-insulin-rich fraction obtained by the gel filtration as a GIT assay substrate.

The amount of mono-FTC-insulin decreased in proportion to the added volume of the rat liver enzyme solution within a range from 20 μl to 80 μl (from 0.4 to 1.6 mg protein). Nonspecific degradation of insulin existed in the assay system was considered to almost completely inhibited by the addition of BSA as a competitive substrate for nonspecific proteases (Fig. 2). Because Tomizawa and Halsey [15], and Chandler and Varandani [6] described that addition of BSA and EDTA completely inhibits non-specific protease digestion of insulin which occurs independently of GSH addition. Time course of insulin degradation by GIT was shown in Fig. 3. Under these conditions, the reaction proceeded lineary up to 5 min, and then rapidly slowed down and apparently came to a stand-still after 10 min incubation. It agreed with that described in previous paper by Tomizawa [14,

Fig. 2. Degradation of mono-FTC-insulin with GIT. 20, 40, 60 and 80 μ l rat liver postmitochondrial fractions were added to FTC-insulin preparation and incubated for 5 min with BSA (●) or without (○). Details were described in MATERIALS & METHODS.

Fig. 3. Time course of degradation mono-FTC-insulin with GIT. Rat liver postmitochondrial fraction (40 μ l) was incubated with FTC-insulins for various periods. Details were described in MATERIALS & METHODS.

15]. Based on these observations, the conditions for the GIT assay were established as described in Materials & Methods.

These facts showed that it was possible to determine the GIT activity by this novel method.

DISCUSSION

In the present study, FTC-insulins were separated

into several peaks by HPLC analysis. According to Bromer *et al.* [3], insulin has three amino acid targets for FITC-I binding; phenylalanine at N-terminal of insulin B chain, glycine at N-terminal of insulin A chain and lysine in B chain. Both phenylalanine and glycine bind with FITC-I under the conditions used in the present study (pH 9.0), but lysine reacts under a higher pH condition (pH > 9.5). Thus, it is considered that two peaks, mono-FTC-insulin and di-FTC-insulin mainly appeared in chromatogram of HPLC analysis. There were many other minor peaks in this substrate, but each of those could not be identified. Fluorescence intensity per protein content in each peak was different. From the ratio, we identified mono-FTC-insulin or di-FTC-insulin molecule. Both molecules were really reduced by the enzyme treatment (Fig. 1); however, there is no evidence that all FTC-compounds are susceptible to the attack of GIT in the same degree.

The reaction products, A and B chains could not be detected by this HPLC method. The molecular weights of A and B chains are much lower than that of insulin. The fact indicate that these products are more susceptible to the digestion with nonspecific proteolytic enzymes than the intact insulin. Thus A and B chains were probably broken into such low molecular weight compounds as could not be detected by HPLC. From the reasons described above, we selected determination of mono-FTC-insulin instead of its products to estimate the GIT activity.

Insulin may be degraded by other proteases [4, 5], in particular, insulin protease and/or nonspecific proteases. The nonspecific proteases could be almost completely inhibited by the addition of excess BSA [7, 16–19]. According to Fig. 2, non-specific degradation seemed to appear in a higher degree when the small amount of enzyme was added to the reaction mixture. Many possibilities could be enumerated; 1) small amount of postmitochondrial fraction included a few molecules of GIT, 2) various proteases which broke insulin were not probably given a simple dose-response curve, 3) some inhibitors might exist in our enzyme source, and 4) these proteases could attack other proteases molecules as substrate at a higher concentration. However, the exact reason was not certain. On the other hand, the insulin protease was disregarded in our method by the following reason.

There are two phases in the insulin degradation process. The primary phase is the insulin cleavage at

the site of its three disulfide bonds by GIT. The products, A and B chains of insulin, are subsequently broken by insulin protease. Although insulin protease can break insulin without GIT, in that case the reaction proceeds very slowly and the insulin degradation is almost negligible within 5 min [7, 8, 17, 19].

Varandani *et al.* determined the GIT activity using whole homogenate in their studies, although they indicated that the GIT activity was highest in the microsomal fraction [17, 19]. In this study, we used the postmitochondrial fraction to assay the GIT activity, as the specific activity of GIT in the fraction is almost same as that in the microsomal fraction [18]. Whole homogenate could not be applied to HPLC as it contains numerous large particles.

Since our method is simpler and more rapid than previous one, we intended to apply it for clinical studies.

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