Labeling polypeptide with ^{99m}Tc and bioactivity get back

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Abstract A method for labeling polypeptide (insulin) with technetium- 99(^{99m}Tc) was established without marked loss of biological activity. Following reduction of intrinsic disulfide bonds by mercaptoethanol and purification on a Sephadex G50 column, the polypeptide was labeled with ^{99m}Tc by transchelation from methylene diphosphonate (MDP). ^{99m}Tc labeled insulin was identified by thin layer chromatograph (TLC) and the change of blood sugar of mice injected, their hypoglycemic shock symptom was also observed. Six hours after labeling, the dissociation of labeled insulin was only 3%, From then on to 24 h, there was no more dissociation. The blood sugar concentration of mice injected with the mercaptoethanol-reduced insulin was $(5.0\pm3.2)\mu mol\cdot L^{-1}$, while those injected with the original insulin was $(1.4\pm1.2)\mu mol\cdot L^{-1}$, the difference was significant (Q test, $p<0.01$). Blood sugar concentration of the mice was $0.3\pm0.2\mu mol\cdot L^{-1}$ for the labeled insulin, and was about the same with that for the original insulin. The labeling efficiency was 74.31% for the labeled insulin, whereas the original insulin cannot be labeled with ^{99m}Tc. The result suggests that while disulfide bonds of polypeptide were reduced by mercaptoethanol, it became free sulfhydryl group, and its bioactivity descended. Then free sulfhydryl group was chelated with ^{99m}Tc under mild condition, restablishing the disulfide bond, therefore, the bioactivity came back. The ^{99m}Tc-labeled insulin was stable during 24 h.

Keywords Peptides, Biological assay, Tc-labeled insulin

CLC numbers O629.7, O621.3+5, Q516, R817.9

1 INTRODUCTION

Recently radiolabeld peptide is being evaluated as diagnostic and therapeutic reagents. It was also reported that increase of affinity and overexpression of insulin receptors is present in hepatocellular carcinomas, breast carcinoma and leukaemia cell as well as several other tumors, therefore insulin can be loaded with radionuclide or other toxic substances to utilize for targeted therapy and targeted diagnosis[1~3]. Direct radiolabeling of monoclonal antibody with ^{99m}Tc has been established by chelation with sulfhydryl group of proteins. However it has been reported that course of labeling of polypeptide opened disulfide bonds of polypeptide, and became a free sulfhydryl group, the change of the ring structure of polypeptide extensively influence its biological activities. Therefore direct labeling method can not be applied for labeling polypeptides[4,5].

Supported by the National Natural Sciences Foundation (No. 30070230)
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In this investigation a method was established which can retain the original bioactivities after radiolabeling of polypeptide with ^{99m}Tc. We devised that disulfide bonds were first opened by mercaptoethanol reduction to become free sulfhydryl groups, then the sulfhydryl group was chelated with ^{99m}Tc which was reduced under mild conditions using Sn^{2+} produced by MDP kit, the disulfide bond was thus reconstructed, and the bioactivity of insulin came back.

2 MATERIALS AND METHODS

2.1 Mercaptoethanol reduction of insulin

20μL purified 2-mercaptoethanonal was added to 1 mL insulin solution (containing 40μ of insulin, approximately 1.538 mg) and incubated for 30 minutes at 25°C with stirring. Mercaptoethanol reduced insulin was purified on a 1.5×10 cm Sephadex G50 column using 0.02 mol·L^{-1} phosphate-buffered saline(PBS) as eluant. The insulin peak was determined by TLC with coomassie brilliant blue and by its function of reducing blood glucose in animal bodies.

2.2 Insulin labeling

Methylene diphosphate (MDP) kit (i.e. 5 mg MDP, 0.5 mg $SnCl_2 \cdot 2H_2O$) was dissolved in 4 mL nitrogen-purged physiological saline. ^{99m}Tc-pertechnetate eluant was diluted with physiological saline to final concentration of 18.5 GBq·L^{-1}. Four vials were prepared under nitrogen, each containing 0.4 mL reduced insulin solution after Sephadex G50 purification (about 0.226 mg), 1 mL (18.5 MBq) of ^{99m}Tc-pertechnetate eluant was added, then immediately MDP solutions of different volume (10μL, 20μL,40μL, and 80μL) were added to each vial respectively. The vials were incubated in 25°C for 5 min. As controls, the identical labeling procedure was applied to the MDP kit + pertechnetate (no insulin, a), pertechnetate only(no insulin and MDP kit, b), and original insulin + MDP kit (c). Then the administrative group(reduced insulin+MDP kit+pertechnetate) was designated as d.

2.3 Labeling efficiency

Labeling efficiency was identified by thin layer chromatograph (TLC) using butanol:ethanol:distilled water=6:3:2 as developing reagents. The radioactivity of each peak on TLC was measured with a gamma counter (Aloka, Japan). The labeling efficiency(percentage) can be calculated by radioactivity of the labeled insulin peak/that of the total TLC.

2.4 Stability of labeled insulin

At 5 min, 3 h, 6 h, and 24 h after labeling, TLC of samples with butanol:ethanol:distilled water=6:3:2 as developing reagents was carried out, dissociation of labeled insulin with

^{99m}Tc was observed.

2.5 Bioactivity of ^{99m}Tc-labeled insulin

Forty male Kunming mice (weighing 20~25 g, first class, License No.24301041, from Experimental Animal Center of Chongqing University of Medical Sciences) were divided into 5 groups, each containing 8 mice. The administration of each group by tail vein was: group A: 0.1 mL saline; group B: 0.1 mL origin insulin; group C: 0.1 mL of 6μ·ml^{-1} mercaptoethanol reduced insulin; group D: 0.1 mL of 6 μ·mL^{-1} of labeling insulin with ^{99m}Tc; group E: no treatment.

Thirty minutes after above treatment, mice were killed by taking blood from eyepit. Concentration of blood sugar was measured with MET-ASCA auto biochemical analyst (MET, USA).

2.6 Statistical analysis

Data are expressed as X±SD. Group differences were determined with the Newman-Keuls multiple range test (Q test).

3 RESULTS

3.1 Reduction of insulin

Molar ratio of mercaptoethanol and insulin was 1000:1[6]. Fig.1 a and b showed the elution curves of insulin and mercaptoethanol reduced insulin on column sephadex G50. The peaks of both were eluted at 9.6 min. The blood sugar concentration of

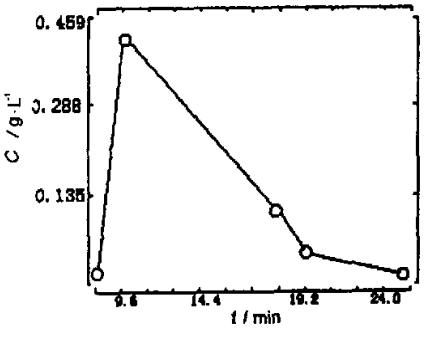

Fig.1a The elution curve of the insulin on Sephadex G50 column, Feed dose 0.1 mL (1.538μmg·L^{-1}); Flow rate 0.625 mL/min; Eluted by PBS 0.02 mol·L^{-1}, pH 7.0.

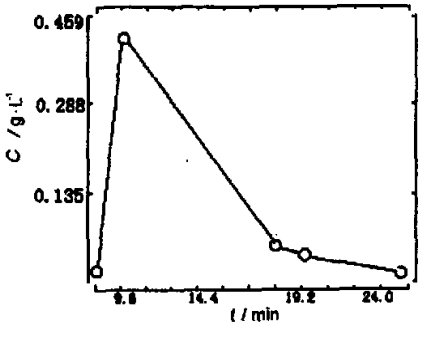

Fig.1b The elution curve of the mercaptoethanol-reduced insulin on Sephadex G50 column, Feed dose 0.1 mL (1.538 μmg·L^{-1}); Flow rate 0.625 mL/min; Eluted by PBS 0.02 mol·L^{-1}, pH 7.0.

mice injected with reduced insulin was $(5.0\pm3.0)\mu mol\cdot L^{-1}$, and was $(1.4\pm1.2)\mu mol\cdot L^{-1}$ for that injected with original insulin. The difference of them was significant (Q test, $p<0.01$).

3.2 Insulin labeling

Fig.2 shows autoradiography of mercaptoethanol-reduced insulin labeled with ^{99m}Tc and three controls developed by TLC. The R_f and ratios of each peak to the total radioactivity are showed in Table 1. The influence of volume of MDP kit on labeling efficiency was showed in Fig.3.

Table 1 Radioactivity ratio of each peak to total: (a) ^{99m}Tc+MDP kit; (b) ^{99m}Tc; (c) original insulin + ^{99m}Tc + MDPkit; (d) ^{99m}Tc + reduced insulin+ MDPkit

Group	Radioactivity ratio of each peak to total		
	$R_f = 0.0 \sim 0.15$	R_f=0.25~0.55	R_f=0.55~0.75
a	70.18		27.02
b			96.74
c	55.54		40.96
d	19.83	74.71	5.46

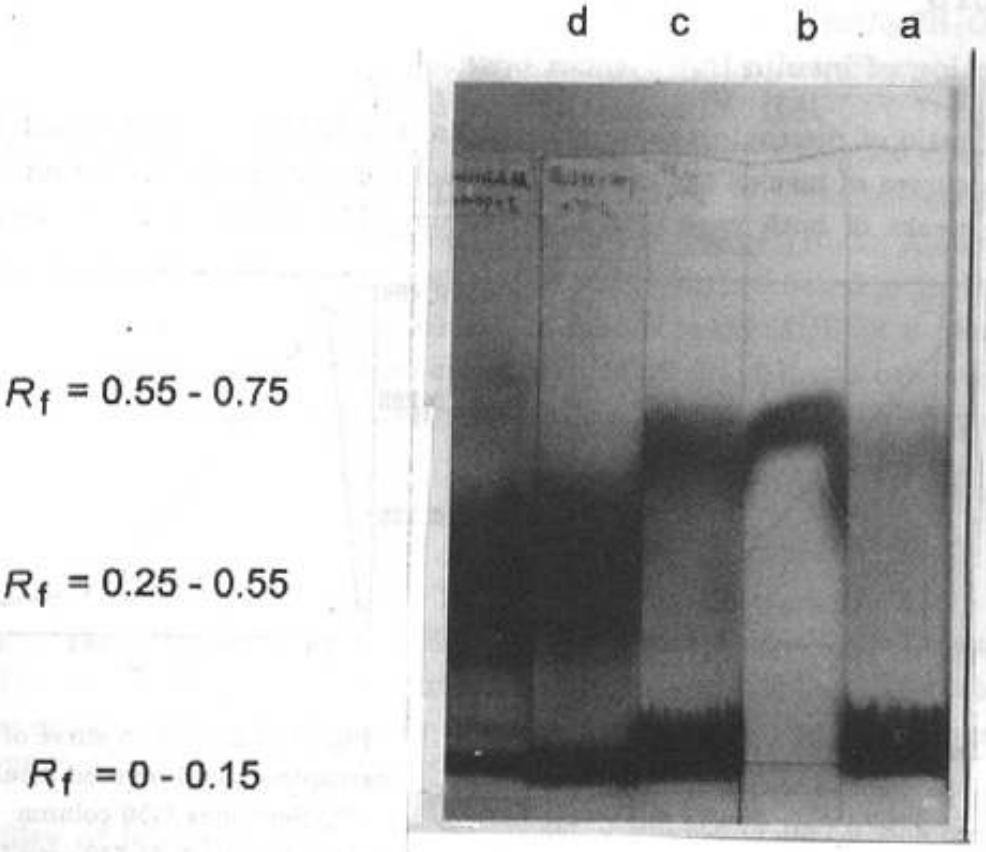

Fig.2 Autoradiography of labeled insulin and controls developed by TLC: (a) ^{99m}Tc+MDP kit; (b) ^{99m}Tc; (c) Original insulin + ^{99m}Tc + MDPkit; (d) ^{99m}Tc + reduced insulin+ MDPkit.

3.3 Stability of ^{99m}Tc labeled insulin

Six hours after labeling, dissociation of the labeled insulin was 3%. From then to 24 h, there was no more dissociation.

3.4 Bioactivity of ^{99m}Tc labeled insulin

The blood sugar concentration of each group 30 min after injection was:

group A:(9.8±1.5)μmol·L^{-1};
group B:(1.3±0.6)μmol·L^{-1};
group C:(5.0± 1.6) μmol·L^{-1};
group D:(0.3± 0.2)μmol·L^{-1};
group E:(8.6± 0.6)μmol·L^{-1}.

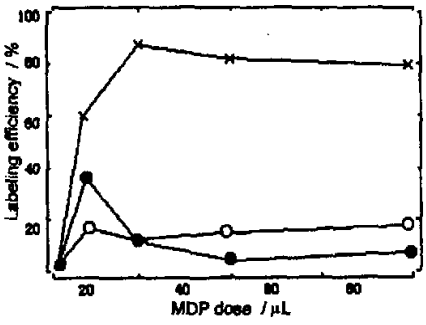

Fig.3 The relationship between the dose of MDP kit and labeling efficiency:
×, ^{99m}Tc labeled insulin, R_f=0.25~0.55;
•, ^{99m}Tc-MDP, R_f=0.0~0.15;
○, free technetium, R_f=0.55~0.75.

4 DISCUSSION

In this investigation, the method of direct labeling monoclonal antibody with ^{99m}Tc reported by Mather[7] was developed, and a method for labeling insulin which have three disulfide bonds was established.

In the mercaptoethanol-reduced insulin, eluant peak of split insulin fragments was not seen after Sephadex G50 purification, the eluant peaks of insulin and reduced insulin were identical(Fig.1). However bioactivity identification of the substances of the two peaks showed that the activity of reduced insulin was lowered greatly. The labeling efficiency of both were also different (Fig.2 and Table 1). It indicated that insulin has been reduced by mercaptoethanol, but chains A and B of insulin were not disconnected completely, only its solid structure was changed, so its bioactivity was lower than original insulin, and eluant peak of split insulin fragments were not seen when were purified on Sephadex G50,. That is to say, only one disulfide bond of chain A and chain B or/and an intrachain disulfide bond of chain A was reduced and disconnected in the reaction.

MDP, as a weak chelator of Sn^{2+}, was oxidized easily. When the dose of MDP kit was small, pertechnetate reducing was not enough. While the dose of MDP kit exceeded some value, MDP chelated more than insulin, the optimal dosage of MDP kit was 20μL.

For identification of the labeled insulin peak of TLC, labeled insulin and three controls were developed on silica gel plates, and were evaluated by autoradiography and were stained with bromophenol blue (Fig.2). The results showed that the free technetium peak was at R_f = 0.55 ~0.75, the peak at R_f=0~0.15 was formed by MDP, the peak of labeled reduced insulin was at R_f=0.25~0.55, but the unreduced insulin was not labeled with ^{99m}Tc. Steigman *et al*[8] described that the labeling reaction of protein

with technetium had two types. One is the strong combination with hydrosulfide group, the other was the weak combination with other amino acid on the surface of molecule, the weak one was not stable. Data of group c and group d suggested that the strong combinations were yielded during labeling insulin by combination of technetium with -SH.

The stability test indicated that the labeled insulin was stable. In 6 h after labeling, dissociation of ^{99m}Tc from labeled insulin was 3%, this was considered as the dissociation of weak conjugation. From then to 24 h, there was not more dissociation, hence the stability could satisfy the requirements of clinical application.

For clinical application, we have to retain the bioactivity of labeled polypeptide with ^{99m}Tc. Results demonstrated that after administration of original insulin or labeled insulin , the blood sugar was lowered significantly, and the hypoglycemic shock symptom was also seen in some mice. Following injecting with mercaptoethanol-reduced insulin, the blood sugar concentration also slightly descended, however, it was much higher than the concentration of the mice administrated with insulin or labeled insulin. It was therefore suggested that the activity of insulin was lowered greatly following the reduction by mercaptoethanol. But when reduced insulin was labeled with 99mTc, its activity returned to normal.

5 CONCLUSION

The result suggests that disulfide bonds of the insulin were broken by mercaptoethanol reduction in labeling, became free sulfhydryl group, and the bioactivity of insulin descended. Under mild condition sulfhydryl group was chelated with ^{99m}Tc, and the bioactivity came back. The ^{99m}Tc labeled insulin was stable during 24 h.

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