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《**Orginal**》

Enzymatic Radioiodination of Insulin for Radioimmunoassay Use

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Abstract

Insulin was labelled with ^{125}I using lactoperoxidase as an oxidizing agent. The reaction product was purified via two stages: a starch gel electrophoresis(SGE) and a Sephadex gel filtration(SF).

Upon comparison of the labelling yields and the bindabilities of the labelled insulin to its antibody, it has been found that the enzyme method shows higher yields (50%) and the better bindability to its antibody than the conventional chloramine-T method (35%).

By checking the insulin blank labelling mixture with a SGE, a paper chromatography, and a radioautography technique, a by-product in the lactoperoxidase method has been identified. The separated fractions in SGE and SF were also analyzed and discussed.

요 약

락토퍼옥시데이즈(lactoperoxidase, LPO)를 사용하여 인슈린을 ^{125}I로 표지반응 시켰다. 반응생성물은 전분젤 전기영동(SGE) 과 세파덱스 젤여과(SF) 등 두단계를 거쳐 정제하였다. 표지수율 과 표지생성물의 항체에 대한 결합능으로 보아 종래의 클로라민-T법(표지수율 약 35%)보다 효소법(표지수율 약 50%)이 우수하였다.

인슈린의 첨가없이 LPO법으로 표지반응을 진행시킨 다음 SGE, 종이 크로마토그래피, 방사선사진술 등을 적용하여 LPO법에서의 부산물을 확인할 수 있었다. SGE와 SF에서의 분리 분별 부분에 관해서도 분석, 토의하였다.

1. Introduction

The recent improvements of radioimmunological assay methods and increasing interest for *in-vivo* hormone kinetic studies[1,2,3] have all led to a deeper research on radioactive tracers. Much effort has been directed towards the preparation of labelled hormones with very high specific activity yet also capable of maintaining the high biological and immunological activities as the native ones. This aspect of the problem is particularly relevant in cases of labelling hormones with radioiodine since the resultant radioiodinated hormone is apt to be different from the original molecule. Chloramine-T (CHT) is most widely used as an oxidizing agent for radioidination of insulin [4,5], but it can lead to over-iodination and

coesequent structural changes of the hormone since with CHT the production of active iodine is so sudden that the reaction is hardly controllable, and such an uncontrollable reaction leads to the over-incorporation of radioactive iodine into the tyrosyl groups of the hormone molecule. On the other hand, lactoperoxidase (LPO) is known as a mild oxidizing agent for radioiodination of insulin since it can produce the labelled insulin having high specific activity[6], moderate stability and adequate immunoreactivity[7~10].

In the preparation and control of insulin radioimmunoassay (RIA) kit using labelled insulin obtained via CHT method, the main difficulty often encountered was the discrepancy of the measured hormone levels (i.e., the large variance of the intra- and inter-assay data), and the cause of which was mainly attributable to the defectiveness of the labelled tracer for the RIA use. Even though some literatures[6~10] concerning the two labelling methods are available, quantitative comparisons such as the labelling yield, purification, identification of by-product, and analysis of the separated peaks by SGE and SF are not much compiled.

2. Experimental

2.1. Enzymatic Radioiodination of Porcine Insulin

The reaction was carried out at room temperature in a small polystyrene tube (11 ×68mm) equipped with a magnetic stirrer and a bar (1×2mm). The reagents were added in the following order[7]; (1) 4 μg porcine insulin, 24.4 IU/mg, Schwarz/Mann, U.S.A., 1 μg/μl, (2) 20 μl 0.4M acetate buffer, pH 5.6, (3) 1 mCi $Na^{125}I$, carrier and reducing agent free for protein iodination, 10mCi/20μl, IMS-300, (or in some cases IM S-30), R.C.C., Amersham, England, (4) 5 μl LPO, 40-50U/mg, Schwarz/Mann, U.S.A., 5μg/μl, (5) 5 μl hydrogen peroxide (300ng) divided into 3 and 2 μl portion; the latter portion was added 1 min. after the addition of the former, (6) 100μl of enzymatic inhibitor; a solution containing 16% sucrose (w/v), 1% potassium iodide (w/v), 0.02% sodium nitrite (w/v), and 0.1% bovine serum albumin (BSA) (w/v). As soon as the reaction was stopped by adding the enzymatic inhibitor, about 1μl of the reaction mixture was spotted on a chromatography paper for an ascending paper chromatography (PC) using 85% methanol as a developing solvent[11], and the whole reaction mixture was subjected to separation by SGE. To check the degree of radioiodination

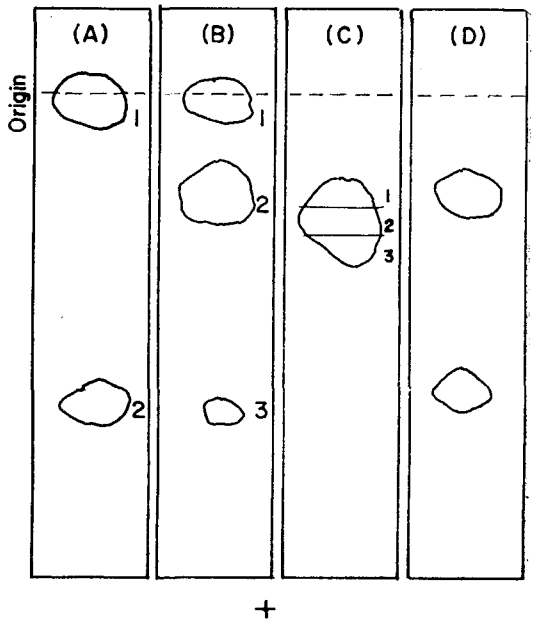

Fig. 1. A Sketch of Autoradiogram of the Starch Gel Electrophoresis Plate
(A) LPO (insulin blank) was labelled by using LPO plus H_2O_2
(B) Insulin was labelled by using LPO plus H_2O_2
(C) Insulin was labelled by using chloramine-T
(D) Insulin was labelled by using H_2O_2

Table 1. Labelling of Insulin by Two Different Methods

Method	Run	Insulin (μg)	Buffer (μl)	^{125}I (μCi)	Oxidizing agent			Reducing agent (μg)	Reaction time (sec)	Reaction temp. (°C)	Labelling yield(%) §		Specific activity of the product (μCi/μg) § § §
					CHT (μg)	LPO (μg)	H_2O_2 (μg)				present work	in ref.[7]	
Chloramine-T (CHT)	1	1	20*	300**	35	—	—	96	6	20	29	51(48~53)	100
	2	1	20	〃	35	—	—	96	10	20	27, 30		
	3	1	20	〃	35	—	—	96	10	23	32, 36		
	4	4	20	800***	4	—	—	25	100	20	42		
Lactoperoxidase (LPO)	5	4	20 #	1000***	—	25	300	100μl # #	120	15	52(22) # # #	52(47~60)	125
	6	4	20	〃	—	25	300	〃	120	20	92(62), 77 (48)		
	7	4	20	〃	—	25	〃	〃	120	22	89(59)		
	8	—	20	〃	—	29	〃	〃	120	20	30 § §		
	9	4	20	800***	—	—	〃	〃	120	20	20		

* phosphate buffer, pH 7.4

** 10,000μCi/100μl

*** 10,000μCi/20μl

\# acetate buffer, pH 5.6

\# \# enzyme inhibitor (see text)

\# \# \# the values in parentheses indicate the net labelling yield of insulin; the labelling yield of LPO(30%) (see run No. 8) was subtracted from the total labelling yield of protein

§ determined by radioactivity counting of the relevant zones after separation by an ascending paper chromatography using 85% methanol as a developing solvent (R_f(insulin)=0.05, R_f (iodide)=0.8)

§ § labelling yield of LPO

§ § § calculated on the basis of the labelling yield, amount of insulin and ^{125}I used for labelling

of LPO itself, 4μg of LPO was added to the reaction tube instead of adding porcine insulin, and the same reaction conditions were followed thereafter. Futher, to check the labelling yield in case of using hydrogen peroxide in the absence of LPO, the same reaction conditions were followed except the addition of LPO (Table 1). Insulin was also labelled with CHT as previously reported[11] for comparison.

2.2. Starch Gel Electrophoresis(SGE)

SGE and autoradiography were carried out as previously reported[11]

2.3. Purification of the Labelled Insulin by a Sephadex Filtration(SF)

The zone **2** fraction of the labelled hormone in SGE (Fig. 1) was extracted from the gel using an eluent buffer (0.02M phosphate buffer containing 2.5% BSA, pH 8.6) and about 0.3ml aliquot of the extract was charged on the top of the Sephadex G-50 column (1×50cm) which was pre-equilibrated with the eluent buffer. The column was eluted with flow rate of about 6ml/hr., and 1ml aliquots were collected in each tube (Fig. 2). The zone **1** fraction of the labelled hormone in SGE (Fig. 1) was also extracted and separated by SF for comparison (Fig. 2).

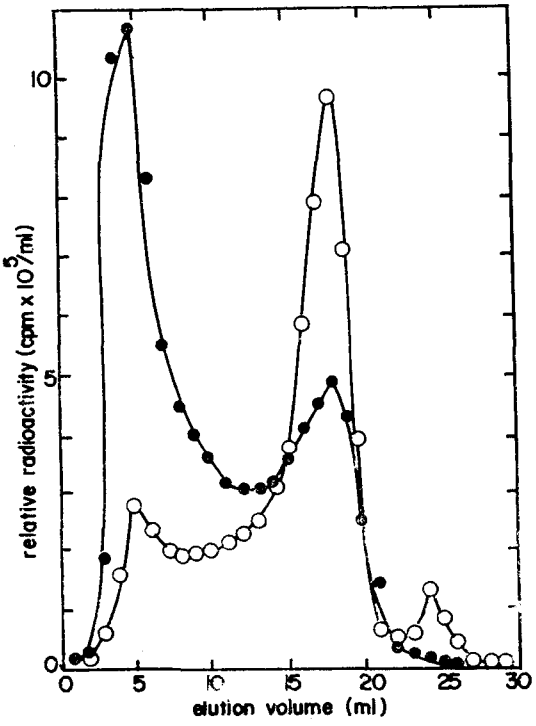

Fig. 2. Sephadex Filtration of the 1 st (—·—) and the 2nd (—∘—) Zone in the Starch Gel Electrophoresis Plate, Fig. 1 (B).

2.4. Measurement of Immunoreactivity (Bindability to antibody)

Each fraction of the SF was incubated with a definite titer of the antiporcine insulin rabbit serum. After incubation for 24 hrs at 4°C, the labelled insulin bound to antibody (B) was separated by using dextran coated charcoal (DCC) suspension from the free unbound labelled insulin (F) as reported previously[11,12]. (Table 2).

Table 2. Adsorption to DCC (%) and Bindability to Antibody (B/F) of the Labelled Insulin*

Method	Fraction (zone) in the SGE plate**	Adsorptivity to DCC(%)	Bindability to antibody (B/F)	Titer of the antibody
Chloramine-T (CHT)	1st	15	0.95	$1:3\times10^4$
	2nd	90	1.87	〃
	3rd	82	0.90	〃
Lactoperoxidase(LPO)	1st	55	0.51	$1:6\times10^4$
	2nd	96	1.57	〃
	3rd	34	—***	〃

* Immediately after the starch gel electrophoresis at 4°C, 300V, for 6 hrs. For bindability determinations, the labelled insulin fractions were incubated with definite antibody titer at 4°C for 24 hrs.

** The order of the located zones from the spotted point (see Fig. 1) in the case of LPO method. In the case of chloramine-T method, the order is the slice number from the origin.

*** Too low to measure

3. Results and Discussion

3.1. Radioidination of Porcine Insulin

The mean efficiency of labelling expressed as the percentage of the total radioactivity

incorporated into the intact radioidinated porcine insulin is about 50% (range; 32-62%) with LPO, and about 35% (range; 27-42%) with CHT (Table 1); The former is nearly consistent with, whereas the latter is a little lower than those in the literature[7]. The cause of the lower yield in CHT method is still unclear but might be attributable to the fact that the trifling factors such as the storage time of $Na^{125}I$ etc. sharply affect the small scale labelling reaction especially in the case of using CHT. It has been confirmed that the labelling efficiency is raised upto 42% with fresh $Na^{125}I$ by applying the CHT method. As Table 1 shows, the mean labelling efficiency is 77% (range 52—92%) with LPO if the labelling efficiency of LPO itself is not subtracted from the total labelling efficiency of protein (calculated from the peak at Rf=O in paper chromatograms). When the labelling efficiency was about 90% by LPO, the net labelling efficiency for insulin was about 60%, and the balance 30% was for the LPO, which is contradictory to the discussions made in the literatures[7,6]. It has been confirmed by carrying out the similar experiment with insulin blank and subsequent paper chromatography using 85% methanol as a developing solvent. The specific activity of the labelled hormone by LPO method is about 125μCi/μg which is slightly higher than that labelled by CHT method. The specific activities were calculated on the basis of the labelling yield, the amount of the hormone and ^{125}I used for labellings. In the adoption of the LPO method but in absence of LPO(i.e.: in the presence of H_2O_2 only), the labelling yield was about 20%. Thus, LPO seems to have the advantage of specifically incorporating the radioiodine into the tyrosyl rings of the molecule in addition to oxidizing iodide to active iodine.

3.2. Purification

As Fig. 1 shows, the autoradiogram of the SGE plate for the labelling mixture obtained by LPO method is similar to, whereas that obtained by CHT mehod is different from those of the literature[7]; the zone intervals are narrow for LPO sample and there was not so much radioactivity remained at the spotted position showing almost a single zone for the CHT sample. The zone **1** and zone **2** in Fig. 1(A) are both originated from the labelled LPO since there is no zone **2** in Fig. 1(B) which is originated from insulin. B.J. Wajchenberg et al,[7] insisted that the separated zones are all originated from insulin by the degree of iodination; the most highly labelled molecules containing diiodotyrosyl residues (DIT) migrate preferentially into zone **3**, whereas the less fully iodinated insulin with relatively greater amounts of monoiodotyrosyl residues (MIT) are found in zone **1**, the same region as unlabelled insulin. However, in refering to Fig. 1(A), the zone **1** in Fig. 1(B) is ascribable mainly to LPO-^{125}I, and the zone **3** in Fig. 1(B) is ascribable to the mixture of the fragmented LPO-^{125}I and the fragmented insulin-^{125}I (vide infra) contradicting to the assignmentmade by Wajchenberg et al. The zone **2** in Fig. 1(B) is ascribable to monoiodoinsulin-^{125}I. Since, the zone **1** and the zone **2** in the SGE was again separated into two to three peaks by the Sephadex filtration (Fig. 2), it is certain that the zone **2** (monoiodoinsulin-^{125}I) is contaminated with the mixture of denatured, and fragmented LPO-^{125}I and denatured, and fragmented insulin-^{125}I due to the inefficiency of separation by the SGE. The small peak appeared at the right side in Fig. 2 indicates

that the zone **2** in Fig. 1(B) is also contaminated with small amount of fragmented LPO-^{125}I and fragmented insulin-^{125}I.

The separation mechanism between the DEAE Sephadex A-25 and Sephadex G-50 would be quite different since the former is a Sephadex (polydextran based) ion exchanger while the latter is a Sephadex filtration matrix of exclusion chromatography. Thus, even if the separation pattern obtained by using the latter material[7] is similar to that obtained by using the former, the peak assignments should not be made similar to those obtained by using the former[6]. The results obtained in present study (Fig. 1 and Fig. 2) strongly indicate that the separation is achieved by the difference of molecular weight (or size) in accordance with a usual gel permeation chromatography (GPC). Since the main constituents of the sample are proteins, the distribution coefficient, K_d, can hardly be larger than 1, and consequently the predominant separation mechanism would be a usual gel permeation[13]. The molecular weight of LPO is 82,000 and that of insulin is about 6,000[14]. It is also known that the denaturation of protein makes the native tight molecule loose and bulky due to the unfolding of the characteristic folded structure[15].

The fragmentation of insulin and some other proteins often occurs at the disulfide linkages.

3.3. Immunoreactivity

As Table 2 shows, the adsorptivity (%) of the labelled insulin obtained by both methods to DCC is roughly proportional to immunoreactivity (bindability to antibody, B/F). The zone **2** in SGE (LPO method) showed the highest immunoreactivity at the antibody titer $1:6\times10^4$. Even though the highest B/F value for the sample (CHT method) is 1.87, the immunoreactivity of the sample is still far lower than that of the sample of LPO method due to the difference of antibody titer. Thus, the LPO method is superior to the CHT method in immunoreactivity point of view.

Generally, the zone **2** (mid-portion) showed the highest B/F values for both samples. The immunoreactivity of zone **3** for the LPO sample was too low to measure under the given conditions.

In summarizing these data (the correlations of peak sizes in Fig. 2(SF), the separated zones in Fig. 1(SGE), and the immunoreactivity relations in Table 2), we assign the zone **1**, **2**, and **3** as LPO-^{125}I, monoiodo insulin-^{125}I, and degraded LPO-^{125}I plus degraded insulin-^{125}I, respectively. These assignments are contradictory to those in the literature[7].

4. Conclusion

Porcine insulin was labelled with ^{125}I using LPO. The labelling yield was about 50%. The formation of a by-product, LPO-^{125}I, has been identified by a series of experiments such as starch gel electrophoresis, Sephadex filtration, and measurements of immunoreactivity.

Relatively pure monoiodoinsulin-^{125}-I fraction could be obtained by a sequential two-stage purification. Thus, the LPO method is superior to the chloramine-T method in both of the yield and the immunological reactivity points of view.

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