ELSEVIER

Journal of Chromatography B, 679 (1996) 21–29

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Labeling of insulin with non-radioactive ^{127}I and application to incorporation of radioactive ^{125}I for use in receptor-binding experiments by high-performance liquid chromatography

Klaus Rissler[a,*], Peter Engelmann[b]

[a]*Pharmbiodyn, Institute of Contract Research, D-79211 Denzlingen, Germany*
[b]*Merial, Gesellschaft für Medizinische und Naturwissenschaftliche Analytik, P.O. Box 1147, D-79332 Herbolzheim, Germany*

Received 5 October 1995; revised 2 January 1996; accepted 8 January 1996

Abstract

Conditions for the labeling of insulin with radioactive iodine isotopes were investigated by means of incorporation of non-radioactive ^{127}I into the peptide. Either the chloramine-T (CT) or lactoperoxidase–hydrogen peroxide (LPO) technique was applied and reversed-phase high-performance liquid chromatography (RP-HPLC) was used for analysis of the reaction products. The LPO method provided the ^{127}I-labeled peptide within 15–30 min, whereas the CT alternative yielded the labeled substrate even within 15 s. However, the latter reaction can only be controlled in a reproducible manner with difficulty and undesired side-reactions became increasingly prominent when the reaction time of 15 s was exceeded for only a few seconds. In another experiment, the LPO technique was applied for radiolabeling insulin with ^{125}I. The product was first purified by size-exclusion chromatography (SEC) and then subjected to RP-HPLC. SEC yielded two peaks. The smaller one, which eluted at a slightly higher K_d value (accounting for about 14% of total radioactivity) predominantly consisted of material eluting at the column's void volume under the conditions of RP-HPLC, whereas the main SEC fraction (accounting for about 86% of total radioactivity) yielded a single peak, as shown by HPLC. The radioactive material attributable to the main SEC fraction revealed the expected receptor-binding properties, as evidenced by displacement experiments with non-radioactive insulin, as well as the action of tetradecanoyl phorbol acetate on the binding characteristics and thus indicating formation of a labeled hormone retaining biological activity.

Keywords: Insulin

1. Introduction

Labeling of target peptides with the radioactive ^{125}I isotope still provides the most efficient procedure to obtain biologically active substances required for receptor-binding experiments and radioimmunoassays. This labeling technique yields high specific activities and counting efficiencies. In this context, the question arises whether radiolabeling extensively preserves biological activity, or instead, exerts a more or less pronounced detrimental effect, which may essentially be associated with the procedure chosen for ^{125}I incorporation. The chloramine-T (CT) method, well-known as the most widely used radiolabeling procedure, provided iodination products, which, depending on the reaction time, contain substantial amounts of impurities at-

*Corresponding author. Present address: K-401.2.08, Ciba-Geigy Ltd., CH-4002 Basel, Switzerland.

0378-4347/96/$15.00
PII S0378-4347(96)00022-9

tributable to undesired side-reactions. These impurities can completely abolish, or at least significantly reduce, the biological potency of the radiolabeled compounds. For this reason, we investigated the formation of the reaction products from the labeling of insulin possessing a rather complex structure, as is often found in a wide variety of biologically active peptide species. In many cases a propensity of such peptide systems to undergo substantial side-reactions after different times of exposure to the labeling conditions is observed. As methods for incorporation of the label, we used the CT as well as the LPO technique. The course of the reactions was monitored chromatographically by means of RP-HPLC. Optimum reaction conditions obtained in this manner were then applied to the labeling of insulin with radioactive ^{125}I, via the LPO method, and subsequent measurement of the polypeptide's receptor-binding properties.

2. Experimental

2.1. Materials

Porcine insulin (lyophilised powder), 12-O-tetradecanoyl-13-acetate (Tetradecanoyl Phorbol Acetate; TPA) and bovine serum albumin (BSA) were products from Sigma (Munich, Germany). Lactoperoxidase (lyophilised powder) was purchased from Calbiochem (Frankfurt, Germany). Chloramine-T (N-chloro-*p*-methylbenzenesulfonamide), potassium phosphate, potassium iodide, sodium chloride, sodium hydroxide, calcium chloride, sodium metabisulfite, triethylamine, hydrochloric acid (36%), orthophosphoric acid (85%), formic acid, trichloroacetic acid (TCA), hydrogen peroxide (perhydrol, 30% in water), all of analytical grade, and glucose (for use in microbiology) were obtained from Merck (Darmstadt, Germany). The [^{125}I]NaI solution, Code-Nr. IMS 30 (diluted in 0.1 *M* sodium hydroxide of approx. pH 13, 0.1 mCi/μl) was from Amersham-Buchler (Braunschweig, Germany). Bacitracin and HEPES were from Fluka (Neu-Ulm, Germany) and fetal calf serum (FCS) and RMPI-1640 medium were from Gibco BRL (Eggenstein, Germany).

2.2. Solutions of reagents

For preparation of the insulin solution, 1 mg of lyophilised powder was dissolved in 800 μl of 10 m*M* hydrochloric acid and reconstituted to a final volume of 1000 μl (1 μg/μl) with 0.5 *M* phosphate buffer, pH 5.0. Purity of the insulin was checked by HPLC at a detection wavelength of 210 nm (0.005 AUFS) prior to use and sample impurities were far below 5% on the basis of calculation of the percentage area. Potassium iodide-127 (16.6 mg) was dissolved in 100 ml of 0.1 *M* sodium hydroxide (0.166 μg/μl$\cong$1 nmol/μl). CT (1 μg/μl), sodium metabisulfite (1 μg/μl) and BSA (7%, w/v) solutions were prepared in 60 m*M* phosphate buffer, pH 5.0. For radiolabeling with ^{125}I, the solution was used as obtained from the manufacturer (0.1 mCi/μl). Furthermore, the original hydrogen peroxide solution was diluted 1:1000 (final concentration 0.03%) with 60 m*M* phosphate buffer, pH 5.0. Lactoperoxidase was dissolved in 60 m*M* potassium phosphate buffer (pH 5.0) and stored in 200 μl portions (25 U/ml)[1] at −30°C. The enzyme can be thawed at least once without marked loss of activity. However, repeated freezing and thawing should be avoided.

2.3. Chromatographic system

The HPLC apparatus consisted of two pulse-dampened LC 410 pumps controlled by a Model 200 gradient controller, an UVIKON 820 UV monitor, all obtained from Kontron Analytics (Munich, Germany), a Valco injection valve equipped with a 100-μl sample loop (Valco Instruments, Schenkon, Switzerland) and a RDK Model 104/102 recorder (Rikadenki, Freiburg, Germany). Separation was accomplished on both a Hypersil ODS (50×4.6 mm I.D., 3 μm particle size) and a Spherisorb ODS 2 column (125×4.6 mm I.D., 5 μm particle size) both obtained from Bischoff Analysentechnik (Leonberg, Germany). Insulin labeled with non-radioactive ^{127}I was monitored at 210 nm, whereas in the case of the ^{125}I-labeled derivative, fractions (1 ml) were col-

[1] 1 U is defined as the amount of enzyme which will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0, at 20°C.

lected and aliquots of 10 μl measured with a type LB 2104 multi-channel radioactivity γ-counter from Berthold (Wildbad, Germany). For isocratic separations of non-radioactive derivatives, 100 m*M* triethylammonium phosphate buffer (TEAP) in 25% acetonitrile (v/v) of an apparent pH of 2.5[2] was used (eluent system 1), whereas gradient elution of radiolabeled peptides was carried out with 100 m*M* triethylammonium formate buffer (TEAF) in 20% acetonitrile (v/v) of an apparent pH of 3.5 (eluent system 2A) and 100 m*M* TEAF in 50% acetonitrile (v/v) of apparent pH of 4.0 (eluent system 2B). For chromatography either in the isocratic or gradient mode, a flow-rate of 1 ml/min was applied.

SEC separation of ^{125}I-labeled insulin was performed on a Sephadex G-50 superfine column (400× 10 mm I.D.), obtained from Pharmacia-LKB (Freiburg, Germany), with 0.1 *M* acetic acid containing 0.05% of BSA, in order to shield the active centers of the gel matrix from unspecific interactions with the radiolabel and was operated under hydrostatic pressure at a flow-rate of approx. 0.3 ml/min. Fractions (2 ml) were collected and the contents measured as described above.

2.4. Labeling of insulin with non-radioactive ^{127}I via the LPO method

A modified iodination procedure as described by Frank et al. [1] was applied at room temperature (RT). To 25 μl of insulin solution (4.4 n*M*), 5 μl of $K^{127}I$ (5 n*M* I≡2.5 n*M* I_2 after oxidation, i.e., the half amount of iodine being available for electrophilic substitution of the aromatic Tyr-moiety) and 5 μl LPO (0.125 U) were added in this range order and quickly mixed on a vortex-mixer. The reaction was started by addition of 5 μl of 0.03% hydrogen peroxide. Aliquots of 10 μl were withdrawn from the reaction mixture after 0.75 min, 15 min, 30 min and 60 min and subjected to isocratic RP-HPLC on a Hypersil ODS column with eluent system 1.

[2]The designation "apparent" relates to adjustment of pH values in the aqueous–organic buffer system by addition of an excess of acid to obtain the desired "apparent" value.

2.5. Labeling of insulin with non-radioactive ^{127}I via the CT method

Labeling was performed at RT according to a modified procedure originally described for the radioiodination of human growth hormone [2]. To 25 μl of insulin solution (4.4 n*M*), 5 μl of $K^{127}I$ (5 n*M* I≡2.5 n*M* I_2) and 5 μl of 0.5 *M* phosphate buffer, pH 5.0, were added and quickly mixed on a vortex-mixer. The reaction was started by the addition of 5 μl of CT solution. Three separate iodinations were carried out and the reactions were terminated after 15, 30 and 60 s by addition of 25 μl of sodium metabisulfite solution under conditions of rapid mixing on a vortex-mixer. Aliquots (20 μl) were injected onto a Spherisorb ODS 2 column. Alternatively, the reaction was stopped by addition of BSA solution (7%, 25 μl) and the products subjected to isocratic RP-HPLC with eluent system 1.

2.6. Labeling of insulin with radioactive ^{125}I via the LPO method

Labeling of 25 μl of insulin (4.4 n*M*) with 10 μl of [^{125}I]NaI (1 mCi) and 10 μl of LPO (0.25 U) was started at RT by the addition of 5 μl of 0.03% hydrogen peroxide solution. After 30 min, the reaction mixture was subjected to SEC on Sephadex G-50 superfine. Fractions (2 ml) were collected with a Frac 200 fraction collector obtained from Pharmacia (Uppsala, Sweden). Aliquots (10 μl) were withdrawn from each fraction for monitoring of the elution profile. Peak fractions from SEC that were attributable to radioiodinated peptide(s) were subjected to analytical HPLC on a Spherisorb ODS 2 column, using a linear gradient with a volatile buffer solution from 100% eluent system 2A to 60% eluent system 2B (i.e., from 20 to 38% acetonitrile) within 30 min. A status of 60% of eluent system 2B was maintained for 15 min followed by a drop to the starting conditions of 100% eluent A within 5 min and a further re-equilibration time of 15 min, after which another sample could be injected. In this manner, 50-μl fractions (approx. $1–2\times10^6$ cpm) eluting between 28 and 37 ml by SEC were investigated by RP-HPLC and subsequent γ-counting. Prior

to the first injection of radioactive peptide, 100 μl of non-radioactive insulin (1 μg/μl) were injected and eluted with eluent system 2, using the gradient profile described above followed by the same gradient but without sample. This procedure was done to saturate deleterious residual silanol groups (minimisation of "silanophilic interactions") for optimisation of ^{125}I-labeled insulin recovery.

2.7. Receptor-binding of ^{125}I-labeled insulin to U-937 cells

The U-937 cells were grown in plastic culture flasks in RMPI-1640 medium containing 10% (v/v) FCS in a humidified atmosphere (95% air–5% carbon dioxide) at 37°C. For receptor-binding assays, cells were grown to a density of about 5×10^6 cells/ml. After sedimentation by centrifugation for 10 min at 300 *g*, cells were resuspended in Krebs-Ringer buffer (assay buffer) consisting of 50 m*M* HEPES (pH 7.5), 150 m*M* sodium chloride, 5 m*M* potassium chloride, 1 m*M* calcium chloride, 10 m*M* glucose, 1 mg/ml BSA and 1 mg/ml bacitracin at 15°C. After two consecutive washes with assay buffer, for complete removal of culture medium, displacement of bound radiolabeled insulin was performed in the same buffer system at a density of approx. 5×10^6 cells/ml and about 20 p*M* of ^{125}I-labeled insulin (≅approx. 100 000 cpm) with increasing concentrations of unlabeled insulin for 2 h at 15°C. Under these conditions about 5000–6000 cpm were bound to the receptor, corresponding to approx. 5% of the total radioactivity. Non-specific binding was determined by addition of 1.5 μ*M* "non-radioactive" insulin. After two washes with assay buffer, binding to cells was measured in a γ-counter. Furthermore, in order to reduce receptor-binding of ^{125}I-labeled insulin, TPA was added at increasing concentrations.

3. Results

The LPO method proceeds with a relatively small reaction velocity, as can be concluded from Fig. 1. After only 15 min of exposure to the labeling conditions, the ratio of peak 1 to the signal of unreacted insulin remained almost constant and did not further increase with time.

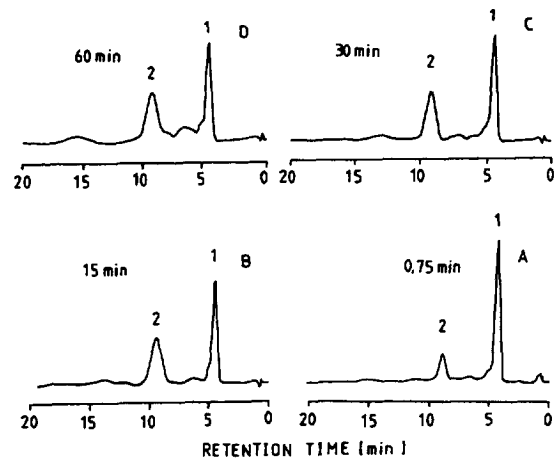

Fig. 1. A–D: HPLC elution profiles of the reaction products by use of the lactoperoxidase–hydrogen peroxide method with ^{127}I at different reaction times (t_R=0.75, 15, 30 and 60 min) on a Hypersil ODS column (50×4.6 mm I.D., 3 μm particle size) with eluent system 1, at a detection wavelength of 210 nm. Peak 1, unreacted insulin; peak 2, reaction product.

In contrast, the insulin peak had completely disappeared after 15 s of exposure to the labeling conditions of CT and a new peak was observed at 15.9 min (Fig. 2A). After a reaction time of 30 s, an additional peak occurred at 31.8 min (Fig. 2B) and

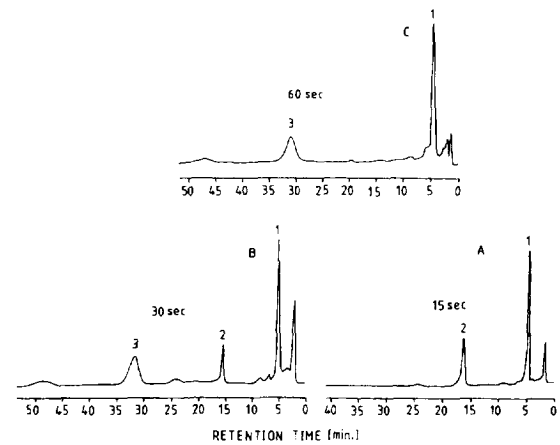

Fig. 2. A–C: HPLC elution profiles of the reaction products by use of the chloramine-T method with ^{127}I at different reaction times (t_R=0.25, 0.5 and 1 min) on a Spherisorb ODS 2 column (125×4.6 mm I.D., 5 μm particle size) with eluent system 1, at a detection wavelength of 210 nm. Peak 1, chloramine-T; peak 2, primary reaction product; peak 3, secondary reaction product. For native insulin, a t_R-value of 8.1 min was observed.

the peak-height ratio of the second and the third peak was approx. 2:1, whereas peak 2 (t_R=15.9 min) completely disappeared after 60 s of reaction, the signal at 31.8 min now represents the only reaction product (Fig. 2C). Under the chosen chromatographic conditions, native insulin elutes at t_R=8.1 min. No marked differences were observed regardless of whether BSA (results not shown) or sodium metabisulfite were used to stop the reaction. This observation indicates that no reductive cleavage of disulfide linkages occurred. The elution profile resembles that of the corresponding LPO iodination, except that the second reaction product is not formed in marked amounts during the LPO-catalysed oxidation. However, in the latter case the individual retention times of corresponding peaks were substantially shifted to higher t_R-values because, for some reason, we were compelled to use a much larger column compared with the short one used for separation of products from the CT reaction.[3]

Chromatography of the reaction product obtained from radioiodination of insulin via the LPO technique on a Sephadex G-50 superfine SEC column yielded two peaks of radioactivity at K_d-values of 0.38 (approx. 86% of incorporated radioactivity) and 0.5 (approx. 14% of incorporated radioactivity), respectively (Fig. 3). Total incorporation of radioactivity into the reaction products was approx. 80% and specific activity was calculated to a value of approx. 300 Ci/g. Only radioactivity recovered at an elution volume (V_e) between 28 and 33 ml exhibited the expected binding characteristics, whereas labeled material at V_e=36–37 ml showed only slight activity. When subjected to RP-HPLC on a Spherisorb ODS 2 column with eluent system 2, pooled fractions eluting between 28 and 33 ml ($K_d \cong 0.38$) yielded a main radioiodination product eluting at t_R=21 min, containing only minor contaminations (<1%, Fig. 4), whereas pooled fractions eluting between 36 and 37 ml ($K_d \cong 0.5$) split into a peak of major intensity (approx. 56% of radioactivity) eluting at approx. 3 min (i.e., at the column's void volume) and a second one of substantially lower intensity (approx. 44% of radioactivity), eluting at 21 min (Fig. 5). Under these conditions, non-radioactive insulin eluted at 19.4 min. Recovery of radiolabeled insulin from the RP-column was >90%. After precipitation of pooled fractions (28–33 ml) with 5% (w/v) TCA, only about 5% of the radioactivity was found in the supernatant.

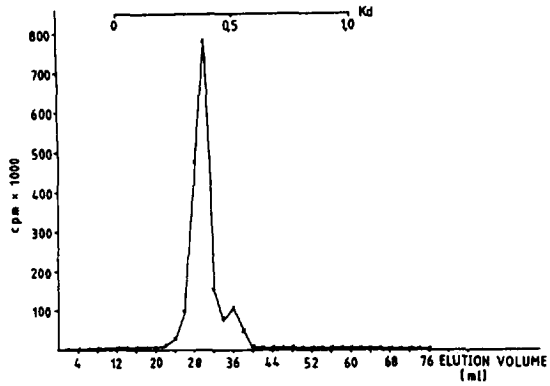

Fig. 3. SEC elution profile of ^{125}I-labeled insulin on a Sephadex G-50 superfine column (400×10 mm I.D.). Fractions (2 ml) were collected, from which 10-μl aliquots were withdrawn for measurement of radioactivity. The K_d values were calculated by the following equation: $K_d = V_e - V_0/V_s - V_0$, where V_e, V_0 and V_s represent the elution volume (obtained at the peak maximum), void volume of the column (determined with dextran blue) and the inner particle volume of the gel matrix (determined with NaCl or [^{125}I]NaI), respectively.

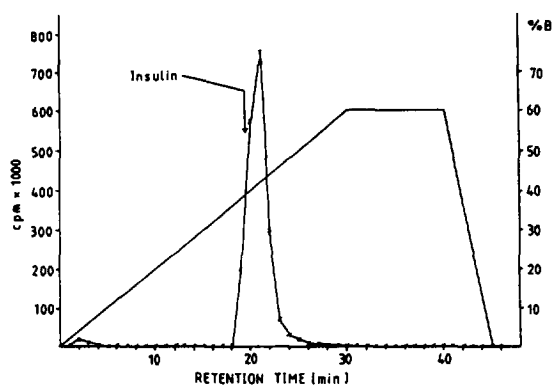

Fig. 4. Gradient HPLC (for details see Section 2 of an aliquot of combined fractions eluting at 28–33 ml on the SEC column (see Fig. 3) on a Spherisorb ODS 2 column (125×4.6 mm I.D., 5 μm particle size) with eluent system 2.

[3] A Spherisorb ODS 2 column was used for the CT experiments because the Hypersil ODS matrix could not be used for completion of the study, due to severe deterioration of the column at the beginning of the CT experiments. A new column of the same type was not available, within the time period of the investigations, thus, the study had to be terminated with another stationary phase.

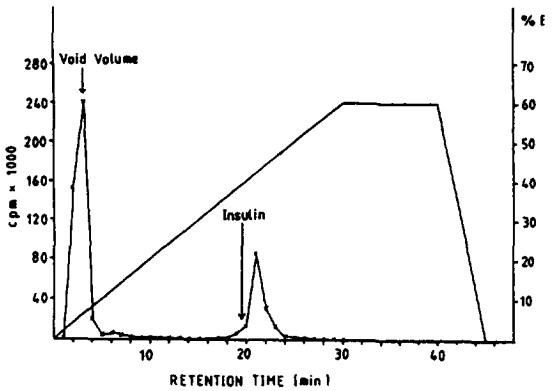

Fig. 5. Gradient HPLC (for details see Section 2) of an aliquot of combined fractions eluting at 36–37 ml on the SEC column (see Fig. 3) on a Spherisorb ODS 2 column (125×4.6 mm I.D., 5 μm particle size) with eluent system 2.

Good binding of ^{125}I-labeled insulin to its receptor located on the U-937 cell line was achieved, as evidenced by the excellent ratio of specific binding (approx. 5000–6000 cpm) versus non-specific binding (approx. 100–150 cpm), respectively. Binding of the ^{125}I-labeled peptide to the insulin receptor using U-937 cells was time-dependent and saturable. Under the chosen experimental conditions, maximal binding of the radiolabel was observed at 260–280 fmol/2×10^6 cells between 90 and 120 min (results not shown). As depicted in Fig. 6, displacement of

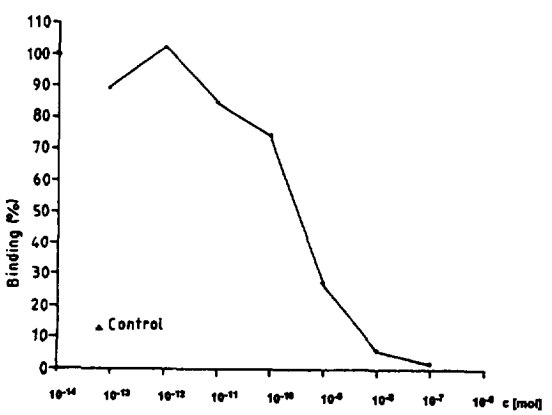

Fig. 6. Displacement curve of ^{125}I-labeled insulin by the unlabeled analogue. The binding assay was performed with approx. 5×10^6 U-937 cells/ml for 2 h at 15°C. Non-specific binding was determined by addition of 1.5 μM unlabeled insulin. The control value expresses binding of the radiopeptide in the absence of its unlabeled analogue.

labeled insulin was half-maximal at about 3×10^{-10} M unlabeled hormone. TPA, known to be a potent activator of protein kinase C, substantially reduced binding of ^{125}I-labeled insulin to about 50% of the initial value, even at a concentration of 10 nM (results not shown). The effects obtained with the radiolabel produced in our laboratory were comparable to those of a commercial preparation of ^{125}I-labeled insulin obtained from Amersham-Buchler.[4] However, binding of the labeled peptide was substantially reduced after removal of the aqueous organic solvent by vacuum centrifugation, when labeled fractions were separated by RP-HPLC in the volatile TEAF system (see Section 4).

4. Discussion

The aim of this study was to develop a rapid method for the monitoring of iodine incorporation into Tyr-moieties of peptides based on analysis of reaction products by RP-HPLC, which could serve as a valuable tool for subsequent preparation of radiolabeled insulin as well as of other peptides, without substantial loss of biological activity.

Insulin as the substrate is composed of two polypeptide chains, consisting of 21 (chain A) and 30 (chain B) amino acids, and has a molecular mass M_r of 5743 Da. Both chains are linked together by two disulfide bridges (Cys^7-A to Cys^7-B as well as Cys^{20}-A to Cys^{19}-B). Moreover, an additional intramolecular disulfide bridge within chain A exists between Cys^6 and Cys^{11}. The polypeptide contains four tyrosine residues (Tyr^{14}-A, Tyr^{19}-A, Tyr^{16}-B and Tyr^{26}-B) and thus, multiple iodination sites are available. This fact includes the possibility that either a complex mixture of insulin species, labeled at different positions, is obtained or that formation of a radiopeptide, which is morefold labeled within the same molecule and, for this reason, is detrimental in subsequent receptor-binding experiments or in radioimmunoassays. Additionally, depending on the reaction conditions, oxidative cleavage of disulfide bridges to cysteic acid derivatives under the labeling

[4]The commercial product was used as received from the manufacturer.

conditions has to be considered. This fact invokes severe deterioration of the biological properties. However, in general, the LPO technique preserves disulfide linkages because only trace amounts of the strong oxidant, hydrogen peroxide, are required. In contrast, iodination with CT involves rather harsh labeling conditions and thus, the possibility of the cleavage of disulfide bridges has to be taken into account when the reaction time exceeds 15–20 s. This observation can be explained by a further increase of the oxidative potency of CT in the presence of catalytic amounts of I^- encountered under the labeling conditions [3,4]. Nevertheless, CT-labeling of insulin was described by Freychet et al. [5] using an equimolar ratio of iodine and CT reagent, but unfortunately no influence of reaction times upon formation of products attributable to undesired side-reactions was reported.

Coupling of [^{125}I]Bolton-Hunter (BH) reagent [4] to free amino groups (e.g., targeted at the N-terminal Gly and Phe as well as Arg^{22} and Lys^{29} residues) was also used to obtain ^{125}I-labeled insulin, with high specific activity. However, formation of either a rather complex mixture of monoiodoinsulins or species having reacted with more than one BH moiety has to be taken into account, thereby decreasing the method's attractiveness, due to the need for extensive chromatographic separation of components. Nevertheless, synthesis of ^{125}I-labeled BH–insulin reacted with three molecules of reagent and extensive conservation of biological action was reported [4].

Two other alternative procedures for iodination include the iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) [6] and the iodo beads variants [7]. The disadvantages of these two methods lie in their low specific activities, and radiolabels prepared in this way should only be used in those cases where the normally gentle conditions of the LPO reaction are nevertheless too harsh.

In general, labeling of Tyr-moieties of peptides is performed with an excess of substrate with respect to the amount of iodine, because it is well-known that incorporation of a second atom of iodine proceeds more rapidly than substitution of hydrogen by the first one [8]. Nevertheless, for labeling experiments with non-radioactive ^{127}I, we used only a slight excess of substrate with respect to the halogen, in order to obtain substantial changes in the peak-height ratio of iodinated product(s) versus insulin as the starting material in the RP-HPLC chromatogram. In this context, the possibility of two-fold incorporation of ^{127}I into the same Tyr residue may, at a first approximation, be considered of only minor importance. Although no amino acid sequence determination of the products and thus no exact evaluation of their precise structure was done, these optimised reaction conditions, yielding only one major component, as revealed by HPLC, were then applied to insulin labeling with radioactive iodine isotopes.

Investigations on the labeling of insulin performed by different research groups with the LPO technique [9–12] revealed that 98–99% of radioactivity was incorporated into chain A and only about 1% into chain B. However, in contrast, a substantially lower amount of only about 85% of ^{125}I was incorporated into chain A and about 15% into chain B under "normal" (non-denaturing) conditions in buffered solution, whereas under denaturing conditions using 3.5 *M* urea, incorporation of ^{125}I into chain A decreased to approx. 55–60%, with 45–50% being introduced into chain B [1]. Under the latter conditions, four peaks observed in the HPLC chromatogram were attributable to the monoiodo insulins labeled at Tyr^{14}-A, Tyr^{19}-A, Tyr^{16}-B, Tyr^{26}-B [1] as also found under similar conditions (6 *M* urea) by Welinder et al. [13]. Concerning the relative distribution of ^{125}I in chain A under normal labeling conditions, 30% was recovered in Tyr^{14}-A and 70% in Tyr^{19}-A, respectively [9]. Furthermore, a dependence of ^{125}I incorporation on the iodination technique was reported [10]. The triiodine method yielded incorporation of about 10% of ^{125}I into Tyr^{14}-A and about 90% into Tyr^{19}-A, whereas in contrast, the LPO procedure favoured iodination of Tyr^{14}-A (approx. 77% in Tyr^{14}-A and about 23% in Tyr^{19}-A). These findings are in accordance with the results obtained by Hamlin and Arquilla [12], but are in marked contrast to those of Lambert et al. [14], who observed a predominant incorporation of ^{125}I into Tyr^{19} of chain A, although the LPO technique was used in both cases. According to Bihler and Morris [15], monoiodotyrosine–insulin consists of a mixture of two species substituted at positions Tyr^{14}-A and Tyr^{19}-A. Furthermore, a marked dependence of Tyr-labeling in either chain A or chain B on the pH value

during application of the LPO procedure was reported [12], yielding a maximum incorporation of approx. 92% of ^{125}I into chain A at a slightly acidic pH. Therefore, it is generally anticipated that enzymatic iodination displays much more specificity towards a single tyrosine compared with non-enzymatic reactions (e.g., ICl or by "in situ" prepared I_2 via CT labeling). This view is also supported by the broad pH range over which LPO exerts its catalytic action for iodination, and thus allows a better "fine-tuning" of iodination conditions.

Due to the fact that the biological activity of iodinated insulin decreases when more than one atom of iodine per molecule of insulin is bound, methods preponderantly yielding monoiodoinsulin are preferred. Iodination at Tyr^{14}-A seems to be less critical, because, in some animal species, this position is frequently replaced by Phe or His, whereas the Tyr^{19}-A position remains constant in all vertebrates. For this reason, it might be assumed that the biological activity would increase as the degree of iodine incorporation at Tyr^{19}-A was decreased.

In our study using sub-stoichiometric amounts of non-radioactive ^{127}I, insulin had not been quantitatively consumed when the LPO technique was chosen, whereas, even after 15 s of reaction, no starting material was present when the CT procedure was applied. This observation is ascribed to extensive degradation of insulin involving cleavage of disulfide bridges and indeed, the product from insulin labeling with ^{125}I via the CT method showed no receptor-binding when, as in our case, the reaction time was extended to 60 s (unpublished results). Therefore, peaks 1 and 2 in Fig. 2B are assigned to the desired ^{127}I-labeled insulin and a degradation product of it, respectively. The fact that consumption of either insulin or formation of the reaction product(s) takes place extremely quickly, makes the CT method unsuitable for labeling because of the difficulty of reaction control.

The latter problems are not encountered by use of the LPO method, and obviously no further processing was observed during reaction times from 15 to 60 min. Excellent control of reaction conditions was achieved and, for this reason, the LPO procedure was chosen for radiolabeling of insulin. Although RP-HPLC of the major SEC fraction eluting at $K_d \cong 0.38$ yields only one peak, it cannot be excluded that polyacrylamide gel as well as capillary zone electrophoresis (not carried out) would have revealed the existence of more than one band. It should also be taken into account that Welinder et al. [13] applied a rather shallow gradient for the separation of the mono-iodinated insulin species and, therefore, our gradient profile eventually may have been too steep for the resolution into two distinct components.

The residual amount of approx. 16% of radioactive material eluting at $K_d \cong 0.5$ and exhibiting only slight receptor binding[5] is ascribed to presumptive degradation products of labeled insulin, which is further corroborated by the results of our HPLC investigations. Elution of the major component takes place at the column's void volume, implying the existence of highly polar components consisting of e.g. cysteic acid derivatives generated by oxidative cleavage of disulfide linkages, despite the use of only low concentrations of hydrogen peroxide by LPO labeling. Thus, on the basis of data from the literature, it is assumed that the fraction of radioactive material eluting at $K_d \cong 0.38$ is attributable to a monoiodinated ^{125}I-labeled insulin derivative. These observations that a single product was already generated with the LPO–non-radioactive ^{127}I system are in good agreement with the results from other research groups [10–13]. Further support of the presence of an intact insulin species is provided by the results from TCA precipitation of fractions 28–33, where only about 5% of the radioactivity remained in the supernatant.

As expected, the phorbol ester TPA exerts a pronounced reduction of ^{125}I-labeled insulin binding, due to substantial phosphorylation of the insulin receptor, and thus additionally confirms the existence of biologically active radiolabeled insulin. Specific interaction of the radiolabeled peptide with its receptor is further substantiated by the concentration-dependent displacement of the radiolabeled peptide by its non-radioactive analogue as well as by the excellent ratio of specific to non-specific binding (5000–6000 versus 100–150 cpm, respectively).

[5] The slight receptor-binding of fractions of radiolabeled peptide eluting at $K_d \cong 0.5$ is ascribed to the overlapping with minor amounts of component eluting at $K_d \cong 0.38$, due to incomplete peak resolution of SEC separation, as evidenced by subsequent RP-HPLC of individual fractions.

Thus, our data are in accordance with those from similar investigations performed by Thomopoulos et al. [16] and additionally underline that the labeling procedure used for preparation of ^{125}I-labeled insulin yielded a hormone retaining its biological activity.

Finally, the observation that the labeled peptide showed reduced receptor-binding to U-937 cells after being subjected to HPLC separation in a volatile buffer system, agrees with the results obtained by Welinder et al. [13] using isolated adipocytes. The authors ascribed this effect to the "unphysiological conditions" of RP-HPLC separation, which are associated with high pressure, shear forces, exposure to damaging organic solvents and the highly hydrophobic column matrix as an additional source of denaturing action.

5. Conclusions

A method for rapid evaluation of labeling conditions with insulin as the substrate is reported. The reaction course of incorporation of non-radioactive ^{127}I, via CT or LPO as the labeling reagents, was monitored by RP-HPLC. The chromatographic patterns obtained after different sampling intervals revealed significant differences between the two techniques, which can be exploited for the choice of optimum labeling conditions with radioactive ^{125}I. The time-course of the CT method exhibited a rapid disappearence of insulin as well as of its primary reaction product, whereas the reaction conditions of the LPO technique proved to be extensively more reproducible, yielding one major component. Application of the labeling conditions obtained in this manner to the preparation of radioactive ^{125}I-labeled insulin derivatives provided a labeled peptide retaining its biological action, as evidenced by its reversible binding to the insulin receptor located on the U-937 cell line. Additionally, the gentle conditions of SEC were sufficient to achieve satisfactory separation of the labeled target molecule from contaminants, as revealed by subsequent HPLC analysis of individual fractions and only one component was found in the main SEC fraction. In contrast, the more or less marked denaturing conditions of RP-HPLC separation invoked a substantial decrease of receptor-binding, but may nevertheless be applicable to shorter peptides, such as, e.g., somatostatin, substance P and others. After minor and substrate-dependent modification in individual cases, this methodological design may be feasible for evaluation of labeling conditions of a wide variety of biologically active peptides.

Acknowledgments

The authors are greatly indebted to Professor Hinrich Cramer (Department of Neurology of the University, Neurochemical Laboratory, Freiburg, Germany) for permission to use his laboratory for preparation and SEC of ^{125}I-labeled insulin.

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