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Solid Phase Synthesis of I^{25} Labeled Insulin Fragments. Part III. Synthesis of 125-I-Insulin Fragment B15-18 Leu-Tyr(I_2)-Leu-Val

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NOWA DAYS, synthesis of biological active peptides has been in an impressive success. This has been achieved by development of new techniques, using new reagents and easy reach.

A new approach to radio-peptides synthesis has been investigated in an effort to overcome the technical difficulties of separation, purification and identification of the prepared peptide fragments. In a series of solid-phase peptide synthesis of labeled insulin fragments, we have prepared 125-I-Insulin fragment Bl 5-18. In this research work we discussed the synthesis of Insulin fragment B15-18 Labeled with Leu-Tyr(I_2)-Leu-Val.

Keywords: Amino acids, Insulin, iodine 125, Labeling, Peptide hormones and Synthesis.

The polymer used in this synthesis was the Merrifield resin[(1)]. The protecting group used for N-terminal was t-butyl oxycarbonyl (BOC).

Cleavage of protecting group has been carried out by IN HCl/AcOH Labeling and identification of the peptide has been achieved by radio-iodination technique using chloramin T. separation and purification carried out by both column and paper chromatography.

This present research study covers the basic idea of the solid phase peptide synthesis[(2)] and radio-iodination technique[(3)] to facilitate the identification and purification of the synthesized tetrapeptide insulin fragment B15-18 to be used for some biological studies[(2,3)].

Experimental

Attachment of Boc- Leucine to the chloromethylated resin

1.52 Gm of Boc-Leu-OH (6.56 millimoles), dissolved in 8.0 ml of absolute ethanol, was added to 4 gm of the chloromethyl polymer (copolystyrene-2%

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divinyl benzene); (5.96 millimoles of CI) suspended in ethanol, followed by 0.92 ml of triethylamine (6.56 millimoles; specific gravity 0.723). The mixture was refluxed for 24 hr.

The resin was filtered and washed, each time, for 15 min with 120 ml portions of ethyl alcohol, water, methyl alcohol and then with methylene chloride and then dried in vacuo.

The degree of substitution of Boc-Leucine on the resin was 0.63 m.mol/gm resin.

Cleavage of Boc group (deprotection from Boc-Leu-O-P)

1.55 Gm (0.98 millimoles) of Boc-Leu-O-P in the reaction vessel, was washed with methylene chloride and glacial acetic acid successively (15 ml, 3 times, 3 min each). Cleavage reagent (1N HCl/acetic acid) was mixed for 5 min (15 ml) followed by another 15 ml for 30 min. The reaction mixture was then filtered off, washed with glacial acetic acid and methylene chlorid successively, (15 ml, 3 times, 3 min each) and dried.

Neutralization took place using 15 ml 10% triethylamine in methylene chloride followed by washing 6 times with l5 ml methylene chloride. The filtrate was found to contain 0.95 millimoles of halogen (Volhard titration), indicating that the deprotection. Process yielded 97%.

Coupling of BOC diiodotyrosine to Leu-O-P

Preparation of Boc- diiodotyrosine

Diiodotyrosine was synthesized according to the procedure followed[(4)]:

A solution of iodine (20 gram) and sodium iodine (26.3 gram) in 88 ml of distilled water was added dropwise with stirring to a solution of L-tyrosine (6.25 gram) in 20% aqueous 'ethylamine (62.5 ml). When the addition was completed, the mixture was stirred for 40 min more. Excess of iodine was removed by treatment with sodium pyrosulphite and the amino acid was precipitated by adjusting the pH to 5 - 6 with acetic acid. The solid was filtered off, washed with water and purified by dissolving in 1 N HCl and reprecipitation with ammonia. Diiodotyrosine melting point was 202 - 205°C, RF: 0.63 (TLC) using BAW system (Butanol[(4)]: acetic acid[(1)]: water[(5)]. The yield obtained was about 73.5%.

Boc-diiodotyrosine was synthesised according to Schnabel procedure, described before using Boc- O-Boc.

The results obtained were found as follows: melting point: 18.6 - 189°C, R_f (TLC): 0.86 using (BAW) solvent system, elemental analysis was: C = 31.51, H= 3.19, N - 2.62 (Calc), C - 31.43, H - 3.21, N =2.58 (Found).

Coupling step

1.1 Gm (2 millimoles) of Boc-diiodotyrosine was dissolved in least amount of dimethylformamide and methylene chloride solvent mixture (15 ml) and added to Leu-O-P in the reaction vessel. Shaking was started for 10 min before the addition of 0.42 grams (2 millimoles) of dicyclohexylcarbodiimide, and then shaking was continued for 6 hr. The reaction mixture was left overnight, filtered off, washed as follows: dimethylformamide (one time), methylene chloride (2 times), absolute ethyl alcohol (5 times) and then with methylene chloride (one time).

Cleavage of Boc group (deprotection) from Boc-Tvr(I2)-Leu-O-P

The protected dipeptide Boc-Tyr(I2)-Leu-O-P was washed in the reaction vessel with methylene chloride and acetic acid successively (3 times, 3 min each time).

15 ml of 1 N HCl/acetic acid was added and shacked for 5 min, filtered and then shacked again-with another portion of 1 N HCl/acetic acid for 30 min. After deprotection step, the reaction mixture was washed with acetic acid 3 times, (3 min each time) then with methylene chloride in the same way. Neutralization was done using 15 ml of 10% triethylamine/methylene chloride with shaking for 5 min, followed by washing 6 times with 15 ml methylene chloride (3 min each time).

The filtrate was found to contain 0.94 millimoles of halogen (Volhard titration), indicating that the deprotection yielded 99% with respect to the last amino acid.

Coupling of Boc-Leucine to the dipeptide Tvr(I_2)-Leu-O-P

0.50 Gm (2 millimoles) of Boc-Leucine dissolved in 15 ml methylene chloride was mixed in the reaction vessel containing the dipeptide Tyr(I_2)-Leu-O-P. After shaking for 10 min, 0.42 gram (2 millimoles) of dicyclohexylcarbodiimide (DCC) was added with continued shaking. The reaction mixture was left overnight, filtered off and then washed as usual using 15 ml of methylene chloride (3 times, 3 min each time), 15 ml of absolute ethyl alcohol 5 times, 3 min each time and then with 15 ml of methylene chloride (one time, 3 min).

Cleavage of Boc group (deprotection) from the tripeptide Boc-Leu-Tyr(I_2)-Leu-P-P(s)

The reaction mixture was washed 3 times with acetic acid (15 ml). Deprotection was performed using 15 ml of N HCl/acetic acid for 5 min, then followed with the same volume of 1 N GHCl/acetic acid for 30 min. After filtration, washing with 15 ml of acetic acid and methylene chloride successively (3 times, 3 min each time) proceeded. Neutralization was performed using 15 ml of 10% triethylamine/methylene chloride for 5 min,

followed by washing 6 times with 15 ml methylene chloride (3 min each time).

The filtrate was found to contain 0.90 miilimols of halogen (Volhard titration), indicating that deprotection yielded 96%.

Coupling of Boc-Valine to the tripeptide Leu-Tyr(I_2)-Leu-O—P

a. Boc-Valine-DCHA was converted to the free acid prior to coupling.
b. Coupling step: 0.44 gm (2 millimoles) of Boc-Valine dissolved in 15 ml of methylene chloride was added to the reaction vessel containing the tripeptide Leu-Tvr(I_2)-Leu-O-P with shaking for 10 min. 0.42 gram (2 millimoles) dicycloclohexylcarbodiimide was then added as coupling agent. Shaking was continued for 6 hr before leaving the reaction mixture overnight. Then it was filtered off and washed as usual.

Cleavage of the finished tetrapeptide Boc-Val-Leu-Tyr(I_2)-Leu-O-P from the chloremethvlated resin

The procedure was explained before in the synthesis of B3-6 and BS-11 fragments[6,7]. The tetrapeptide was got yielded 0.76 gram (82.6%) with respect to the first amino acid (Leucine) attached to the resin. Its melting point was 136-140°C; with respect to amino acid analysis it was shown in Table 1.

TABLE 1. R_f values of I^{125}-peptide fragment [insulin (B15-18)] as shown by different methods; using (BAW) synthesis.

Spotted samples	Rf values	
	Color	Radio scanning
First peak of I^{125}-peptide	0.661 0.912	0.660 0.911
Second peak of 1^{250}- peptide	0.912	0.910
Third peak of I^{250}-peptide	0.553 0.919	0.551 0.918
Fourth peak of I^{250}- peptide	0.570	0.571
Fifth peak of I^{250}-peptide	0.848	0.850
I^{125} as NaI carrier free used in labeling	0.850	0.852
+ Baw synthesis: Butanol (4): Acetic acid (1): Water (5).		

Purification

Purification process was discussed in details in [6,7].

Radioiodination of Insulin (B15-18) fragment [Boc-Val-Leu-Tyr(I_2)-Leu-OCR3]

13.60 mg of insulin (B15-18) fragment was radioiodinated by the procedure described before[6,7].

Fractionation of the labeled fragment on sephadex G- 50 column, showed the following results:

In relation to the radioactivity assay of eluted fractions (Fig.l, there are five peaks).

On the other hand, Fig.2 related to spectrophotometry at wavelength 254 un for each fraction, showed five peaks.

By comparing peaks obtained from Fig. l and 2 we found that the second peak in the two figures is our main products while the other peaks are contaminate products.

Paper radiochromatography scanning (Fig. 3) for the separated peaks showed that some peaks contain minute amounts of other components with Rf values calculated from the diagram, we got (Table 1).

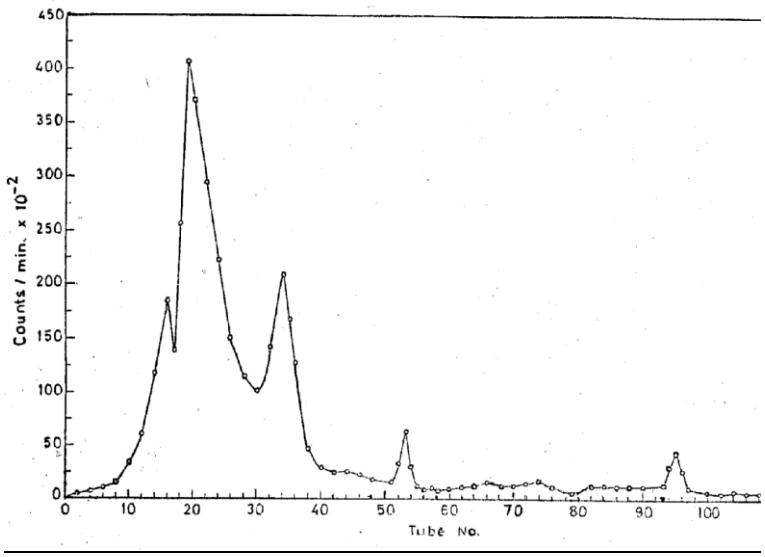

Fig. 1. Fraction of I^{125} labeled peptide fragment (Insulin (B_{15-18}) on sephedex G - 50 column. Radioactivity Assey of Eluted Practions.

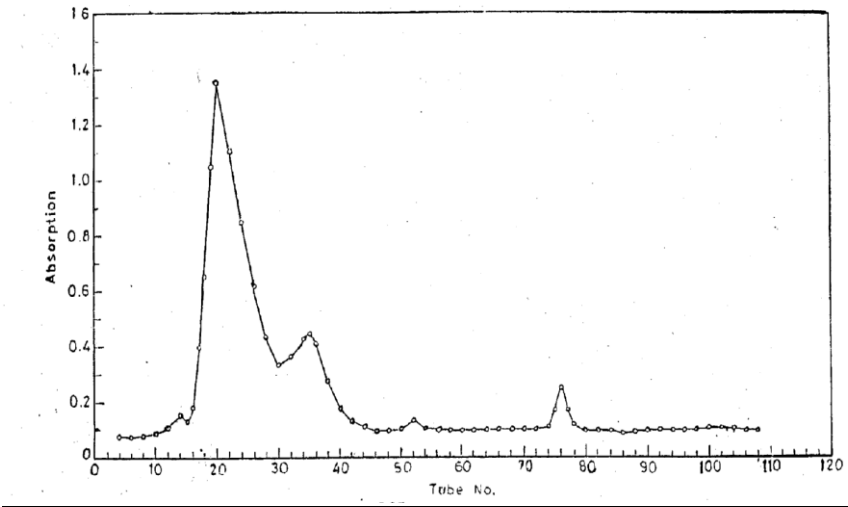

Fig. 2. Fraction of I^{125} labeled peptide fragment (Insulin (B_{15-18}) on sephedex G - 50 column. Spectrophotometery Assey at Wave Length 254nm.

Radioiodination of insulin (B15-18) fragment

From the fragment sequence, the only possible position to be iodinated was B16 which contains tyrosine residue. Tyrosine radioiodination proceeds in which the iodine atoms substitute ortho to the hydroxyl group in the phenolic ring of tyrosine[5].

After fractionation on column chromatography using sephadex G-50, radioactivity was measured giving rise to five peaks (Fig.l) from which the second peak was confirmed to be our pure peptide possessing higher radio-activity. Also, on measuring the ultraviolet absorption for each fraction at wavelength 254 nm, we got five peaks (Fig. 2) from which the same peak (second peak) was confirmed to be the main fragment.

Evaluation of R_f values obtained from paper radio chromatography scanning and those from paper chromatography on our preparation and the standards (Tables 1 and 2) we concluded the following:

- The first peak (a) is our main peptide contaminated with diiodotyrosine.
- The second peak (b) is our main peptide.
- The third peak (c) is our main peptide contaminated with small peptide.
- The fourth peak (d) is small peptide.
- The fifth peak (e) is earner free I^{I25} as NaI^{125}.

To ensure these results, amino acid analysis (Table 3) was performed from which we found that the second peak is exactly our main peptide.

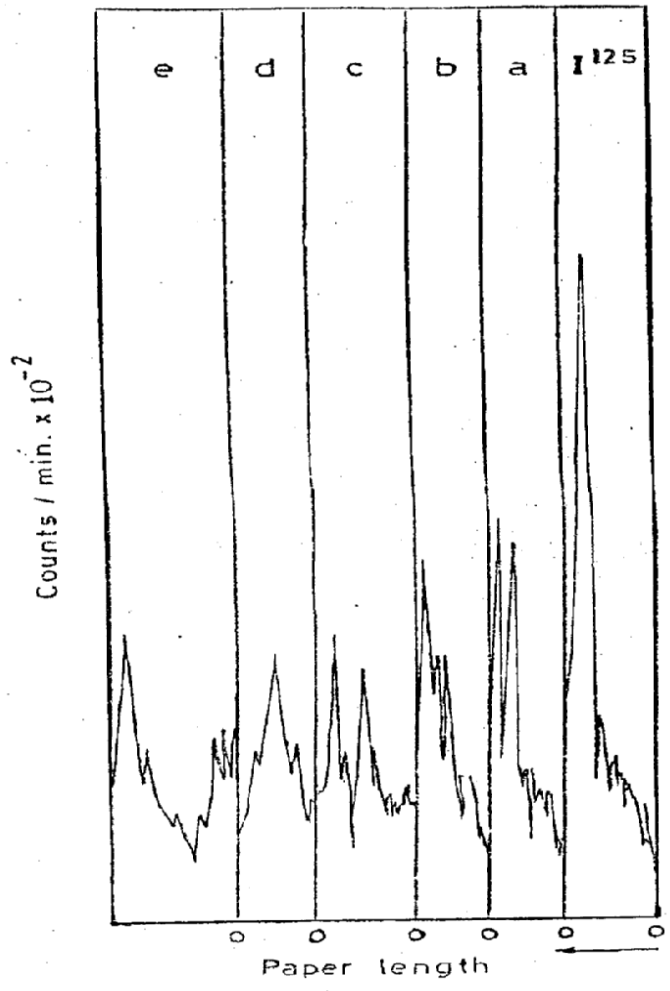

Fig. 3. Paper, Radiochromatography scanning for the separated peaks of 1/125 labelling of the peptide fragment [insulin (B_{15-18})].

a= peak No.1 **b= Peak No. 2**
c= peak No.3 **d= Peak No. 4**
e= peak No.5

TABLE 2. R_f values of standard amino acids and synthesized peptide fragment [insulin (B15-28)], under the same system.

Amino acids and (B15-18) fragment	Fragment (B15-18)	Leucine	Diiodotyrosine	Valine
R_f Values	0.922	0.620	0.660	0.4541

TABLE 3. Amino acid analysis for the synthesized peptide fragment [Insulin (B15-18)].

Amino Acid Analysis	Leucine	Diiodotyrosine	Valine
Theoretical	1	1	1
Found	2	0.7	0.5

References

1. **Merrifield, R. B.,** *I Am. Chem. Soc*. **86**, 304 (1964).

2. **Merrifield, R.B. and Corigliano, M.A.,** *Biochem. Preparation*, **12**, 98 (1968).

3. **Freedlender, A. and Cathou, R.E.,** P.94 In: *Radioimmunoassay Methods*, edited by Kirkham, K.E. and Hunter, W.M., Edinburg, Churchill Livingstone, 689 (1971).

4. **Barnes, JJ-L, Borrows, E.T., Elks, J., Hems, B.A. and Long, A.G.,** *J. Chem. Soc.* **4**, 2824 (1950).

5. **Zewail, M.A.,** *Bull. NRC, Egypt*, **6**, 163 (1981).

6. **Megahed, Y.M., Abd El Wahab, M.R, Zewail, M.A. and Shalaby, A.M.,** *Isotopenpraxis* **19,** 8, 274 (1983).

7. **Zewail, M.A., Abdel-Wahab, M.R., Megahed, Y.M. and Shalaby, A.M.,** *Isotopenpraxis* **22**, 6, 218 (1986).

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تخليق الببتيدات المكونة لأجزاء الانسولين "سلسلة ب" بإستخدام طريقة التصنيع على سطح البلمرات (ثلاثى بيوتيل اوكى كاربونيل)- فالين- ليوسين- ثنائى أيود والتيروزين- ليوسين (ميثيل استر)- ب 15-18 (السلسلة ب للإنسولين)

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الاساس العام لتخليق الببتيدات بإستخدام طريقة السطح الصلب تعتمد اساسا على ارتباط الحمض الامينى الاول من السلسة براتنجات صلبة بواسطة روابط تساهمية يبدا بعدها إضافة باقى الاحماض الامينية المتتابعة فى خطوات متتالية و تدريجيا حمض امينى واحد فى المرة الواحده الى ان نصل الى الترتيب المطلوب ثم يفصل الببتيد المصنع من على السطح الصلب

و يحتوى هذا البحث على تخليق جزء من السلسة ب للإنسولين بإستعمال هذا الطريق و هو كالأتى : جزء ب 15-18 من السلسة ب للإنسولين-(ثلاثى بيوتيل اوكى كاربونيل)- فالين- ليوسين- ثنائى أيود والتيروزين- ليوسين (ميثيل استر)