DAEHAN HWAHAK HWOEJEE
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2-브로모프로피오닐화된 수지를 이용한 인슐린 A(1-21) 사슬의 합성

鞠淳雄† · 洪南注*
고려대학교 이과대학 화학과
*영남대학교 농축산대학 응용미생물학과
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Synthesis of Insulin A(1-21) Chain and Their Assembly on a Polymer-Bound α-Methylphenacylester Linkage

Soon Uoong Koock and **Nam Joo Hong***
Department of Chemistry, Korea University, Seoul 132, Korea
**Department of Applied Microbiology, Yeungnam University, Daegu 632, Korea*
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요 약. 2-브로모프로피오닐화된 수지를 이용해 insulin A(1-21) 사슬을 합성하였다. 시스테인의 결사슬은 각각 acetamidomethyl, benzyl, 그리고 benzhydryl 기로 보호하였으며 그루타민과 아스파라긴은 p-nitrophenyl 기로 활성화하여 합성에 이용하였다. 매 짝지음단계마다 DCC/HOBT coupling agent로 각 아미노산을 축합하였으며 반응의 완결여부는 닌히드린시험으로 측정하였다. 생성물은 NH_3/MeOH-Dioxane(v/v 1 : 1)로 수지로부터 분리하여 DEAE-Sephadex A-25와 Sephadex LH-20으로 정제하였으며 최종생산물은 HPLC, electrophoresis로 확인한 결과 순수한 것(>99%)으로 나타났으며 총수득율은 6%이었다.

ABSTRACTS. The total synthesis of insulin A chain (1-21) with properly protected sulfhdryl groups of three cysteins for the correct intra and inter disulfide bond formation has been accomplished on 2-bromopropionylated 2% DVB-styreneresin support employing manually operated rotary vessel. The sulfhydryl groups of cysteins were protected with acetamidomethyl, benzyl, and benzhydryl respectively. Glutamine and asparagine were attached to the peptide chain by active ester coupling, all other amino acids were coupled with DCC/HOBT. The synthesized peptide was purified by DEAE Sephadex A-25 and gel filtration Sephadex LH-20. The final product was found to be homogeneous by HPLC, electrophoresis, and amino acid analysis. The overall yield of the pure isolated peptide was 6%.

INTRODUCTION

The polypeptide hormone insulin is produced in the β-cell of the islet of the pancrease. The physiological signal for the synthesis and secretion of this hormone is increased by bloodglucose level. Insulin influences the whole of intermediate metabolism. It accelerates glycolysis, the pentosphosphosphate cycle and the synthesis of glycogen in the liver. Futhermore, insulin

promotes protein biosythesis and fatty acid synthesis. Insulin deficiency causes diabetes mellitus, metabolic malfunction disease characterized by hypercaemia, glycosuria and tendency to hetosis.

Sine the discovery of the primary structure published in 1958 by Sanger, Katsoyannis et al[1]. successfully synthesized the Achain of sheep insulin in 1963. Katsoyannis described the total synthesis of sheep insulin and the total synthesis of human insulin by solution method in 1966. Hisky[2] was succesful in the synthesis of a protecd insulin A-chain which contains intact intrachain disulfide bridge in 1972 for the first time. The cysteine side chain 7 and 20 were protected by benzhydryl(Bzh) and trityl groups. Some polymer supported segment condensation of A chain were known[3~6] but total stepwise solid-phase synthesis of A-chain has not been reported so far.

As part of effort devoted toward the synthesis of large petide, the protected insulin A (21 amino acid residues) has been synthesized by solid-phase stepwise method at our laboratory. Because of its size, well-characterized protein, and the verification for the possiblity to synthesize large peptides with rotary reactor which was designed in our laboratory[7], insulin A was selected as model compound.

The synthesis followed the stepwise strategy with the acidlabile tert-butoxycarbonyl (t-Boc) group for the temporary N-protection of the amino acid, benzyl(Bn) group for hydroxyl protection of serine and γ-carboxyl of glutamic acid, 2, 5-dichlorobenzyl(2, 5-DCB) group for hydroxyl protection of tyrosine, p-nitrophenyl (O-NP) group for the activation of a carboxylic acid of glutamine and asparagine. Acid sensitive Bzh, acid stable acetamidomethyl(Acm) and Bn group was selected for the protection of sufhydryl group of cysteine.

stepwise synthesis had been performed on the 2-bromopropionyl resin to investigate the potentiality for the use of this resin in the synthesis of large peptide as well. Cesium salt method[8] was employed for the purpose of the first anchoring of amino acid. Dicyclohexylcarbodiimide(DCC) and 1-hydroxybenzotriazole(HOBT) were simultaneously used as coupling agents.

EXPERIMENTAL AND PROCEDURE

Materials. t-Boc-N_3 was prepared from phenylchlorochloroformate and t-butanol[13]. t-Boc-amino acids were synthesized by the method of Schnabel.[9] Copoly(styrene-2% divinylbenzene) resin(fluka), 200-400 mesh, 1.23 μmol of Cl/g was selected as solid-support. TLC was performed on silica 0.250mm, 60F 254 (Merk) in the following system(v/v): A, 1-butanol/acetic acid/water 70:15:15 B, 2-butanol/acetic acid /ethylacetate 85:5:10 C, ethylacetate/pyridine/ acetic acid/water 25:20:6:10. The TLC plate was stained both with ninhydrin and chlorine/ tolidine reagents. The preparative chromatography was performed on Sephadex LH-20(2.2 ×40cm) in methanol and acetonitrile solution and also DEAE Sephadex(2×120cm) in ammoniumbicarbonate(pH 8.1) buffer solution.

Preparation of 2-bromopropionyl Resin. 2-Bromopropionyl chloride(5g, 24mmol) was added slowly to a suspension of 4g of aluminium chloride in 50 ml of dichloromethane with gentle stirring. The brownish solution was added to a suspension of copoly(styrene-2% divinyl benzene) resin(20g, 200-400 mesh) in 220 ml of dichloromethane. The mixture was stirred for 17hrs. The acylated resin obtained was collected and washed successively with dicholomethane, nitrobenzene, and THF. The slightly brownish resin was collected by filtration. The operation was repeated once more and the resin was washed with water, THF

and then MeOH: IR(KBR) 1685 cm-1[10](C=O), Volhard titration[11] of Br indicated Br 0.92 meq./g.

Protection of L-cysteine. N-Hydroxylacetamide was prepared with 83% yield, m.p. 50°C from acetamide and 35% formaldehyde in basic medium.[14] S-Acetamidomethyl-L-cysteine(Acm-Cys-OH) hydrochloride was prepared by treatment of L-cysteine with N-hydroxymethlacetamide in hydrochloric acid at pH 0.5. The usual procedure[9] was used to prepare Boc-Cys(Acm)-OH from t-Boc-N_3. NMR and IR spectra were consistent with proposed structure. Yield 52%; $[\alpha]_D^{25}$ −35.8(C=1 in H_2O); Rf 0.81 (A); m.p. 109°C.

Treatment of dipenylmethanol with *L*-cysteine hydrochloride in acetic acid containing boron trifluoride etherate com plex provided the Bzh-Cys. Another protecting method[15] was also performed with TFA as catalyst. The former gave some better yield. The method could be utilized carbinol as potential cation for alkylation of sulfur. The usual procedure[9] was used to prepare Boc-Cys (Bzh)-OH from t-Boc-N_3. NMR and IR spectra were consistent with the proposed structure. Yield 81%; $[\alpha]_D^{25}$ +6.4 (C=1 in chloroform); m.p. 157°C.

Preparation of L-Aspargine *p*-Nitrophenylester. DCC is highly efficient for solid-phase peptide synthosis, but it suffers from the disadvantage of over-activation. Therefore, the amide side chains could participate in the intramolecular side reaction and dehydration[9]. To avoid this problem, Boc-Gln-Onp was synthesized by the method of Schnabel. IR spectrum was consistent with the proposed structure. Yield 45%; Rf 0.79(A); $[\alpha]_D^{25}$−55(C=1 in DMF); m.p. 144~145.5°C.

Protection of tyrosine. 2,6-DCB group was used for the protection of side chain in tyrosin by method of Yamashiro et al[12]. IR and NMR spectra were consistent with the proposed structure. Yield 63%; $[\alpha]_D^{25}$+21(C=1 in ethanol); m.p. 110°C.

Preparation of t-Boc-Asn-OCs. t-Boc-Asn-OCs was synthesized from t-Boc-Asn-OH by the method of Koock et al[7].

Synthetic protocol. The following are based on 1g of starting resin and the routine liquid volumes were 15ml. The synthetic protocol was as *Table* 1 and 2.

RESULT AND DISCUSSION

Solid-Phase Synthesis Procedure. At first the heneicosapeptide was built up on 1g of bromoacetophenylated 2% cross-linked poly styrene resin(1.23meq. of Cl/g). All polymer supported reation were carried out in a manually operated rotary vessel throughout the schedules shown in *Table* 1 and 2. All coupling except that of Boc-Asn-OH and Boc-Gln-OH were carried out by DCC couping method using 3 eq. of Boc-amino acid, 3eq. of DCC in dichloromethane and 6 eq. of HOBT as an additive to suppress racemization[16]. Boc-Asn-OH

Table 1. Schedule A for TFA deprotection-DCC/HOBF coupling in solid-phase synthesis

step	Reagent	Vol (ml)	Time (min)
1	DCM wash(3 times)	15	5
2	30% TFA-DCM	15	1.5
3	30% TFA-DCM	15	30
4	DCM wash(6 times)	15	9
5	5% DIEA-DCM	15	1.5
6	5% DIEA-DCM	15	1.5
7	DCM wash(6 times)	15	9
8A	3eq. Boc-A.A. in DCM	10	1.5
8B	3eq. DCC-6eq. HOBT in DMF	10	120
9	Recouple if necessary by repeating steps 4-8		
10	DCM wash(3 times)	15	5

DIEA; diethylamine, DCM; dichloromethane, HOBT; 1-hydroxybenzotriazole.

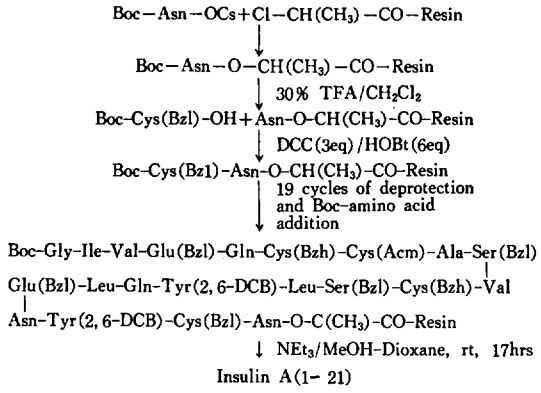
Table 2. Schedule B for TFA coupling-active ester coupling in solid-phase synthesis

Step	Reagent	Vol (ml)	Time (min)
1	DCM wash(3 times)	15	5
2	30% TFA-DCM	15	1.5
3	30% TFA-DCM	15	30
4	DCM wash(6 times)	15	9
5	5% DIEA-DCM	15	1.5
6	5% DIEA-DCM	15	1.5
7A	DCM wash(6 times)	15	9
7B	DMF wash(3 times)	15	4.5
8	Boc-amino acid active ester in DMF(3eq.)	10	300
9	Recouple if necessary by repeating step 4-8		
10	DMF wash (6 times)	15	9
11	DCM wash(3 times)	15	5

and Boc-Gln-OH were coupled by active ester method in DMF which was synthesized from p-nitrophenol. Since N^{α}-Boc protection was used throught the synthesis, a suitable selection of side-chain protecting groups was required. Bn group for side chain protection was used to glutamic acid and serine. And also Bn, Bzh, and Acm groups were chosen for cysteine protection. To form the dipeptide on polymer, "reverse DCC coupling" method[17] was employed for the second amino acid coupling. To determine the extent of the coupling, ninhydrin test[18] was performed before every coupling step.

Almost all negative tests were obtained after double coupling procedure. The unreacted amino groups were blocked by acetylation with a 1 : 1 (v/v) mixture of acetic anhydride and pyridine. Acetylation procedure[11] had been required at valine and cysteine coupling steps. Assembly of the heneicosapeptide sequence was outlined in Scheme 1.

Attachment of first amino acid to the 2-bromopropionylated resin support. Cesium salt of the first Boc-amino acid was attached to the 2-bromopropionylated polymer support.



Scheme 1.

Thus, the cesium salt of Boc- Asn-OH was allowed to react with the resin in DMF at room temperature in a manner similar to that described by Gisin[8] for the standard procedure of attachment. Boc-Asn resin phenacylester was obtained with good substitution, 98% yield. IR spectrum indicated the NH of amide stretch band at 3400cm^{-1} and the carbonyl of ester at 1720 cm^{-1}.

Cleavage of the synthetic insulin A(1-21) from the support. Polymer supported heneicosapeptide(212mg) was treatedwith 40ml of triethylamine in methanol/dioxane(1 : 1 v/v) for 17hrs. The released product was separated by suction. After evaporation of the solvent, the resulted oily residue was crystallized in ether. The yield was 51mg. The product contained only minor impurities as shown in HPLC analysis(*Fig.* 1).

Isolation and purification of the product. The crude product(10mg) was dissolved in 2m*l* of o.1M ammonium bicarboate buffer solution (pH=8.1) and placed on a DEAE Sephadex A-25 column(2×120cm) equilibrated and eluted with the same buffer. The sample in the colum eluate was monitored continuously with a LKB Bromma recording sepectrophotometer at 275nm. The chromatographic pattern obtained(*Fig.* 2) indicated the presence of one major component,

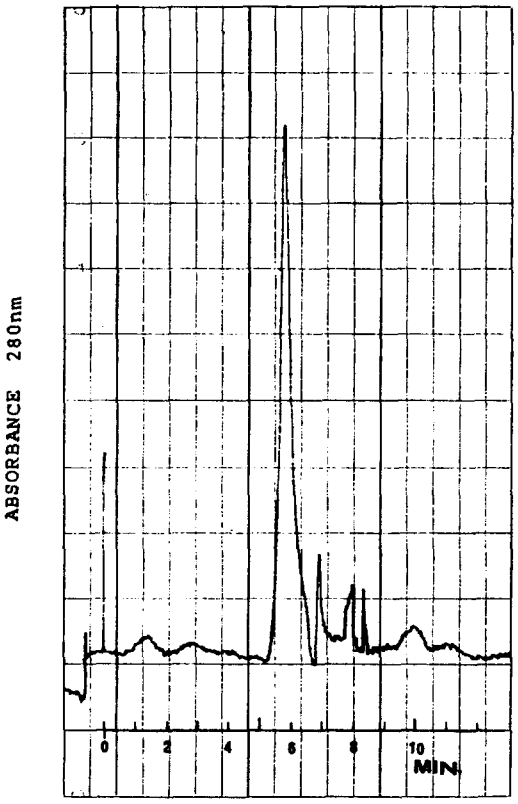


Fig. 1. HPLC analysis of crude insulin A(1-21) derivatives. Conditions: μ-Bondapak colum(4×300 mm), detection at 280nm, solvents of phosphate buffer in 5% MeOH/water, 0.08 absorbance scale, sampl injection 10μl, flow rate 1.0ml/min, pressure 860psi.

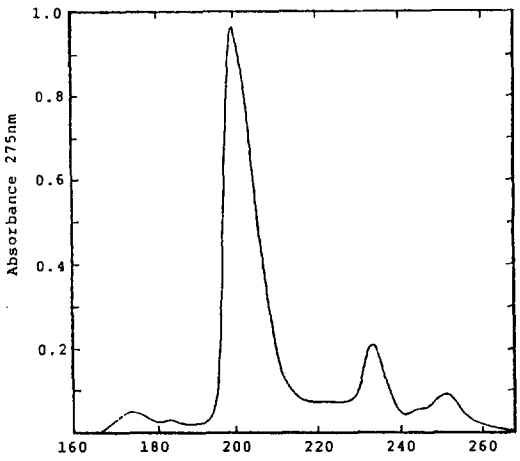


Fig. 2. Anion-exchange cromatography of crude product on diethylaminoethyl Sephadex A-25. The column(2×120cm) was equilibrated with 3.3M ammonium bicarbonate buffer(pH 8.1) solution.

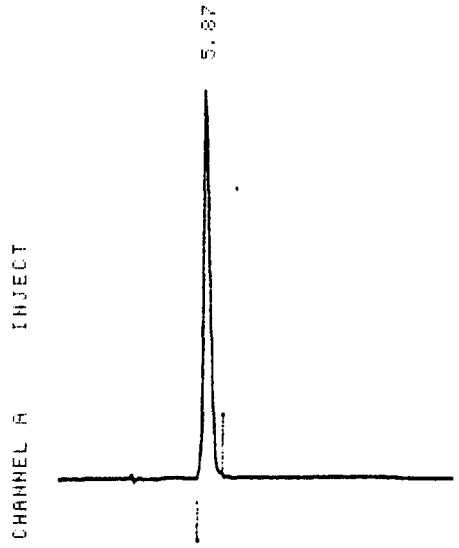


Fig. 3. HPLC analysis(the identical conditions of Fig. 1) of the purified product after Sephadex LH-20 gel filtration.

the insulin A chain and only small amounts of other conponents. The eleate under the major peak between 195 and 210ml was collected and then placed in a benzoylated dialysis tubing and dialyzed against four change of distilled water (2l, each) at 4°C for 24hrs. The dryed product was dissolved in 2ml of CH_3OH/CH_3CN(3 : 1 v/v) and applied to a Sephadex LH-20 column (2×40cm) equilibrated and with the same solvent mixture. The eluate under the almost single major peak was collected and evaporated. The purity of the resulted product(>98%) was analyzed by HPLC(*Fig.* 3). The overall yield from the two column was 5.9mg(59%) at this stage.

Amino acid analysis after total hydrolysis

Table 3. Amino acid analysis of sythetic insulin A (1-21) sample

Amino acid	Found	Theoretical
Asp	2.1	2
Ser	1.8	2
Glu	4.1	4
Gly	1.0	1
Ala	1.1	1
Cys	3.6	4
Val	1.9	2
Ile	1.0	1
Leu	2.0	2
Tyr	1.9	2

Dried samples of the protein were hydrolyzed with 12 N HCl/ACOH/phenol(2 : 1 : 1 v/v) for 24hrs at 110°C, and the amino acids were quamtitated on Hitachi 835 amino acid analyzer.

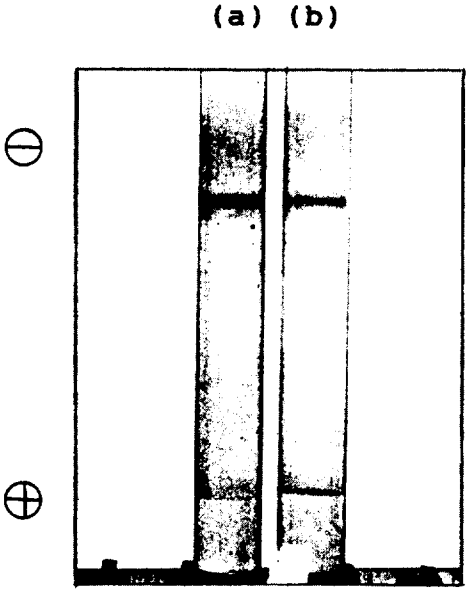


Fig. 4. Gel electrophoresis of natural(a) and synthetic(b) insulin A chain on polyacrylamide gel at pH 8.3. Detection by staining with Coomassie-Brilliant Blue R-250.

with 12 N HCl/AcOH/phenol(2 : 1 : 1 v/v) for 24hrs at 110°C gave a composition in good agreement with the theoretically expressed value(*Table* 3). Gln and Asn were changed to Glu and Asp in this hydrolysis step. TLC gave a singl spot(Rf 0.73(A), m.p. 260°C decomposition). The IR spectrum gave the amide carbonyl stretch absorption at 1660cm^{-1}. And also, the NH overtone band at 1560cm^{-1}.

Polyacrylamide gel electrophoresis in the presence of 0.1mol Tris-glycine buffer was perfomed at pH 8.3(0.6×8.5cm) by utilizing 2~30% polyacrlyamide linear graldient disc gel. Synthetic and natural insulin A(0.2mg) were dissolved in 200μl 0.1mol phosphate buffer (pH =7.5). Electrophoressis was performed at 100V for 1hr and then 200V for 7hrs. After the destaining, the one single band indicated that any other impurities were not present. Electrophoretic pattern of synthetic material was also identical with that obtained with natural hormone(*Fig.* 4). The overall yield of the pure isolated protein was 6%.

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