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Solid phase synthesis of the signal sequence of *E. coli* alkaline phosphatase

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Abstract. A synthetic peptide corresponding to the signal sequence of *E. coli* alkaline phosphatase has been synthesized by the solid phase method employing the transesterification method of cleavage from the resin. The protected peptide obtained after cleavage was purified to homogeneity by column chromatography on silica gel, followed by partition chromatography on Sephadex LH-20 using organic solvents like chloroform and methanol as eluants.

Keywords. Signal sequence; solid phase synthesis; transesterification; partition chromatography.

1. Introduction

Proteins destined for export in eukaryotic and prokaryotic cells are synthesized as precursors with amino terminal extensions (signal sequences) varying between 15–40 residues (Silhavy *et al* 1983). The signal sequences of secretory proteins are characterized by a positively charged region followed by a 15–20 residue stretch of apolar amino acids (Watson 1984). The precursor regions of mitochondrial proteins are much larger and apolar and charged residues are distributed uniformly (Hay *et al* 1984). Extensive studies using biochemical (Walter *et al* 1984), recombinant DNA (Lingappa *et al* 1984; Duffaud *et al* 1985) and genetic (Bankaitis *et al* 1985; Benson *et al* 1985) techniques have revealed the following facts: (i) there is no primary structure homology among signal sequences (ii) introduction of a charged amino acid in the hydrophobic region renders a signal sequence non-functional (iii) signal sequences can initiate export of cytoplasmic proteins and also target them to organelles like chloroplasts and mitochondria (iv) proteins that recognize signal sequences are components of the cells' export machinery.

In spite of the large amount of information available in the intracellular sorting of proteins, questions like, what are the common recognition elements in signal sequences? and how do signal sequences actually initiate translocation of polypeptide chains across membranes? are still unanswered. Structural and other physico-chemical studies on signal sequences which should help in answering these questions have been few (Briggs and Gierasch 1984; Katakai and Iizuka 1984; Laxma Reddy and Nagaraj 1985; Rosenblatt *et al* 1980; Shinnar and Kaiser 1984) presumably due to the difficulties in obtaining signal sequences in sufficient amounts. An approach based on chemical synthesis is the only way to generate signal sequences, as isolation from precursor

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proteins is technically very difficult (Ito 1982). In fact, the few structural studies that have been reported are based on synthetic signal peptides. In an effort to generate signal sequences in sufficient amounts for structural studies, which are amenable to post synthetic modifications, we had explored the transesterification method of cleavage in the solid phase synthesis of signal peptide fragments (Laxma Reddy and Nagaraj 1985) and obtained protected peptides in good yields which could be easily purified by column chromatography on silica gel employing organic solvents. In this paper we describe the synthesis of a 20-residue peptide corresponding to the signal sequence of *E. coli* alkaline phosphatase (Silhavy *et al* 1983) shown below, employing the transesterification method of cleavage.

Lys-Gln-Ser-Thr-Ile-Ala-Leu-Ala-Leu-Leu-Pro-Leu-Leu-Phe-Thr-Pro-Val-
1 5 10 15
Thr-Lys-Ala
20

2. Experimental

2.1 *Materials*

Amino acids, dicyclohexylcarbodiimide (DCC), diisopropylethylamine (DIEA), 1-hydroxybenzotriazole (HOBT) and chloromethylated polystyrene co 1% divinyl benzene (200–400 mesh, 1·03 m eq/gm) were purchased from the Sigma Chemical Co, Mo, USA. Trifluoro acetic acid (TFA) was from Fluka, Switzerland. Protected amino acids were prepared by established methods (Stewart and Young 1984). Dichloromethane (CH_2Cl_2) was distilled over P_2O_5 and Na_2CO_3. DIEA was distilled over ninhydrin. The protecting groups employed were *t*-butyloxycarbonyl (Boc) for α-NH_2 groups, benzyloxycarbonyl (Z) for ε-NH_2 group of Lys and benzyl (Bzl) for the OH of Ser.

2.2 *Assembly of the peptide chain*

The first amino acid (Ala at the 20th position) was attached to the resin by the cesium salt procedure of Gisin (1973). A substitution of 20% i.e. 0·2 mmol of Ala for 1 gm of resin was used for the synthesis. Substitution was estimated by the picric acid method (Gisin 1972). The Boc amino acids of Ser (OBzl), Thr, Ala, Phe and Val were coupled as symmetric anhydride which were prepared as follows: the Boc amino acid (1·2 mmols) was dissolved in CH_2Cl_2 cooled to 4°C and DCC (1·2 mmols) added. After 15 minutes at 4°C, the DCC urea was filtered off and the CH_2Cl_2 solution of the Boc amino acid symmetric anhydride was used for coupling. The Boc amino acids of Leu, Ile, Pro and Lys (ε-Z) were coupled with DCC. Boc Gln was coupled with DCC and HOBT in DMF/CH_2Cl_2 2:8 (*v*/*v*). Each coupling was performed twice. After the addition of Ala at the 16th position, each coupling was performed thrice. The couplings were monitored by the picric acid test (Gisin 1972). Solid-phase reactions were carried out on a mechanical shaker in a glass vessel equipped with a fritted disc and a stop cock. One cycle of synthesis based on 1 gm of starting resin consisted of the following operations: (1) CH_2Cl_2 wash, 15 ml, 3 × 1 min (2) deprotection, 20% TFA/CH_2Cl_2, 15 ml, 30 min (3) CH_2Cl_2 wash, 15 ml, 6 × 2 min (4) prewash, 5% DIEA/CH_2Cl_2, 15 ml, 1 × 2 min

(5) neutralization, 5% DIEA/CH_2Cl_2, 15 ml, 1 × 10 min (6) CH_2Cl_2 wash, 15 ml, 6 × 2 min (7) equilibration with Boc amino acid, 0·6 mmols, in 10 ml CH_2Cl_2, 10 min or Boc amino acid symmetric anhydride, 180 min (8) DCC in 5 ml CH_2Cl_2, 180 min when symmetric anhydride method of coupling was not employed (9) 30% ethanol/CH_2Cl_2 wash, 15 ml, 3 × 2 min (10) CH_2Cl_2 wash, 15 ml, 3 × 2 min (11)–(14) were similar to operations (7)–(10). After step (14), chain termination was effected by acetylation with acetic anhydride:pyridine:benzene 1:3:3, 15 ml, 2 × 10 min. The coupling time for Boc Gln was 24 hours. At the end of the synthesis, the resin was washed with methanol and dried. The weight of the resin at the end of the synthesis was 1·3 g. About 100 mg of the resin was consumed for the amino acid analysis and the picric acid test during the course of the synthesis.

2.3 *Cleavage from solid support*

Cleavage of the peptide from the resin by transesterification was achieved as follows: a suspension of the peptide-resin (0·65 g) in 30 ml of anhydrous methanol and 4 ml of triethylamine was stirred under reflux for 4 hours. The resin was filtered and the methanol solution evaporated to yield the crude peptide. Three cycles of transesterification were carried out to ensure complete recovery of the peptide. From 0·650 g of resin, the amount of crude peptide obtained was 0·100 g.

2.4 *Purification by chromatography on silica gel*

The crude, fully protected peptide (80 mg) was first purified by column chromatography on silica gel (30 × 2 cm). The sequence of elution was as follows: (1) 200 ml $CHCl_3$ (2) 200 ml 2% MeOH/$CHCl_3$ (3) 200 ml 4% MeOH/$CHCl_3$ and (4) 1000 ml 5% MeOH/$CHCl_3$. About 400 fractions of 4 ml each were collected. Thin layer chromatography (tlc) profiles in 10% MeOH/$CHCl_3$ (peptides visualized by starch/KI) indicated that fractions 231–325 were similar. These fractions were pooled and evaporated to yield 20 mg of peptide which gave the following amino acid analysis on a LKB 4151 alpha plus analyzer after hydrolysis in TFA/HCl 1:2 in vacuum for 24 hours: Ser 0·55 (1), Lys 1·66 (2), Glx 0·64 (1), Thr 2·71 (3), Ile 0·50 (1), Ala 2·90 (3), Leu 4·58 (5), Pro 1·82 (2), Phe 1·00 (1), Val 1·20 (1). Values in parentheses indicate theoretical values. Since the amino acid analysis was not satisfactory and tlc indicated heterogeneity, the peptide was further purified by partition chromatography on sephadex LH-20 (Anggard and Bergkvist 1970).

2.5 *Purification by partition chromatography*

20 g of Sephadex LH-20 was refluxed in MeOH for 14 hours. The resin was filtered and air dried for 12 hours. The dry resin was allowed to swell in 30% MeOH/$CHCl_3$ for 2 hours. The peptide (20 mg) was dissolved in 0·5 ml 30% MeOH/$CHCl_3$, applied to the column (bed dimensions: 60 × 1 cm) and eluted with 30% MeOH/$CHCl_3$. Fifty fractions of 1 ml each at a flow rate 0·1 ml/min were collected. Pure peptide (homogeneous on tlc) was obtained in fractions 24–28. The remaining fractions (29–32) were pooled and the impure peptide was again purified in the same manner. The total yield of pure peptide was 10 mg.

The protecting groups were removed by treatment with TFA/thioanisole (Bodanszky

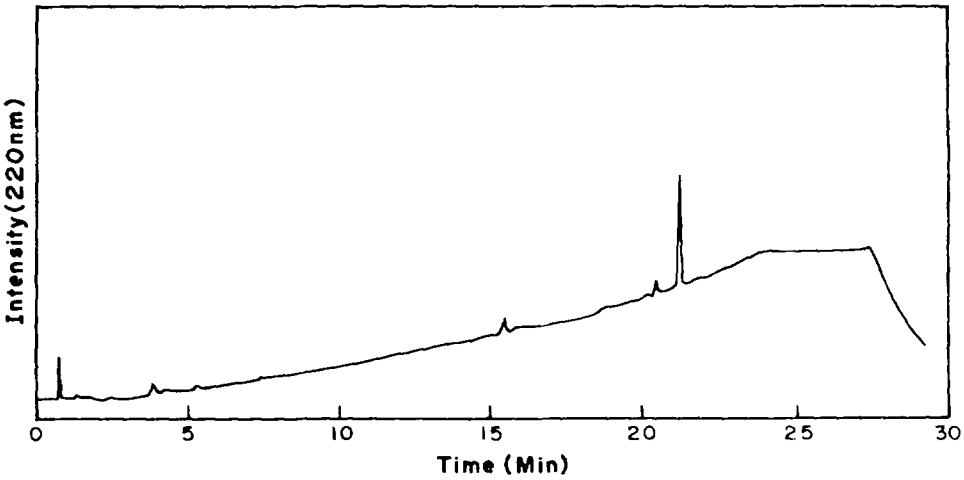

Figure 1. High pressure liquid chromatographic analysis of the synthetic *E. coli* alkaline phosphatase signal peptide. Conditions: solvent A = 50% MeOH/H_2O, solvent B = 0·05% TFA in MeOH, linear gradient of 0–100% B into A in 30 min, 0·25 ml/min; detection at 220 nm.

and Bodanszky 1984) and chromatographed on a small sephadex LH-20 column. The purified deprotected peptide was analysed by high pressure liquid chromatography on a Hewlett Packard C-18 microbore column (100 × 2·1 mm, 5 μM) in a Hewlett Packard HP 1090 instrument and was found to be homogeneous (figure 1). Amino acid analysis after hydrolysis in TFA/HCl 1 : 2 for 24 hours (Shinnar and Kaiser 1984) in vacuum was as follows: Thr 2·02 (3), Ser 0·59 (1); Glx 1·00 (1); Ala 3·17 (3); Val 1·00 (1); Ile 1·02 (1), Leu 5·19 (5); Phe 1·01 (1); Lys 2·20 (2); Pro 2·11 (2). Numbers in parentheses are theoretical values. The TFA/HCl method is known to yield low recovery of Thr and Ser as a result of partial degradation of these amino acids.

3. Results and discussion

The synthesis of the *E. coli* alkaline phosphatase signal peptide has been achieved on a solid support employing well established protocols for solid-phase peptide synthesis (Stewart and Young 1984). However, cleavage of the peptide from the solid support was accomplished by transesterification, a method not commonly used now-a-days. Although cleavage by anhydrous HF is extensively used in solid-phase peptide synthesis (Barany and Merrifield 1980) considerable difficulties were experienced in the purification of a signal peptide obtained by this method (Rosenblatt *et al* 1979). Apart from generating protected peptide suitable for post synthetic modifications for physico-chemical studies, the transesterification method of cleavage also aids in purification, as the protected peptides show good solubility in organic solvents. Since most signal sequences have amino acids with short side chains like Gly, Ala or Ser at the carboxy terminus, steric hindrance is unlikely to reduce yields in the transesterification reaction. Hence, in the solid-phase synthesis of signal sequences, cleavage by transesterification would be more advantageous than by other methods commonly employed.

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