Peptide Research

Simulation of Continuous Solid Phase Synthesis: Synthesis of Methionine Enkephalin and its Analogs

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ABSTRACT

The method of continuous solid phase synthesis, i.e., synthesis performed on a continual carrier, transferable from one synthetic compartment to another by means of a mechanical device, allows one to perform multiple synthetic steps simultaneously on different regions of the carrier. This procedure was tested on the synthesis of methionine enkephalin and its analogs. Products, obtained in reasonable yields, were fully characterized. Possible arrangements and the use of continuous solid phase synthesizers are discussed.

INTRODUCTION

The high demand for synthetic peptides, stimulated by results of immunologists characterizing particular areas of proteins as potential vulnerable targets for "magic bullet" drugs, has led to the development of several techniques for rapid and multiple peptide syntheses. Examples of these synthetic procedures are: Synthesis in "tea bags" by Houghten (5); synthesis on grafted polyethylene pins (4); synthesis on paper (3); and multiple continuous flow synthesis performed in a series of columns (6). However, all these methods have certain disadvantages, such as limited size of the synthetic compartment (tea bags), low physical stability of the carrier (paper), or extremely low capacity (pins). It has been shown (2,3) that paper can be successfully used for the synthesis of peptides in varied amounts, the limit being only the mechanical stability of the support. Cellulose beads were tested as a solid support for peptide synthesis by Merrifield in 1963 (8), but were found to be inferior to polystyrene resin.

Ideally the carrier should be compact in structure, variable in size, easily separable into well defined parts, mechanically and chemically stable, and compatible with the conditions of peptide synthesis. Any polymeric material in the form of a thin membrane, containing suitable functional groups (polystyrene, grafted polyethylene or tetrafluoropolyethylene, polyamide, etc.), may fulfill some of these requirements, but often the mechanical properties are not appropriate. We have found that cotton sheets satisfy most of the criteria given above.

Having found cotton sheets to be a suitable carrier, we examined their application to continuous solid phase synthesis. Up to now, all peptide syntheses, either in solid or liquid phase, had to be performed in batches, since there was no way to continually supply the carrier to the solution containing the activated amino acid or deprotecting mixture and, at the same time, continually obtain carrier on which one of the synthetic steps was performed. The carrier, which is in the form of a continuous strip, may proceed through the particular synthetic or washing steps, bringing the peptide at the end of its pathway through the various compartments either bound to the carrier or dissolved in the solution used for its cleavage.

Since construction of a complete machine for continuous solid phase synthesis is a rather difficult task, we decided to test the basic idea first using a simple model system simulating the continuous synthesis.

EXPERIMENTAL

Synthetic Setup

Synthesis was performed with the use of only one shaker bath (5 x 5 cm) and a mangle machine. The latter consisted of two counter-rotating Teflon cylinders (7 cm long, 2.5 cm in diameter), which were pressed together with adjustable springs. The speed of rotation could be set from 1-30 cm/min. The cotton strip was removed from the bath and introduced between the cylinders together with a Whatman 3MM paper strip which was *ca.* 1 cm wider than the cotton strip. The moist cotton strip issuing from the mangle was introduced to the bath containing the next solution. The strip was sequentially passed through the following solutions: EtOH (2 min), DCM (2x2 min), 20% piperidine in DMF (20 min), DMF (2x2 min), DCM (2x2 min), Fmoc-amino acid, DCC, HOBt in DMF spiked with bromophenol blue (7) (all components, 0.2 M, coupling time according to the color of the carrier – 0.5 to 3 h), DMF (2x2 min), EtOH (2 min).

Modification of the Cotton Strip

A cotton strip (width 3 cm, length 280 cm, weight 32.1 mg/cm^2) was shaken for 15 min in a mixture of trifluoroacetic acid (25 ml) and dichloromethane (75 ml), washed by dichloromethane (3x100 ml), 10% DIEA in dichloromethane (2x100 ml), dichloromethane (3x100 ml) and dried *in vacuo*. This pre-treated strip was then shaken in a mixture of Boc-Gly (4.4 g), dicyclohexylcarbodiimide (5.15 g), dimethylaminopyridine (3.05 g) and dimethylformamide (100 ml) for 4 h at room temperature. After washing by DMF (3x100 ml), ethanol (3x100 ml), and DCM (3x100 ml), the carrier was dried. A sample of the carrier (1 cm^2) was shaken in a solution of trifluoroacetic acid (25%) in DCM, washed by DCM (3x), 10% DIEA in DCM, DCM, and then treated with Fmoc-Gly, DCC, and HOBt in the presence of bromphenol blue (7) until the blue color disappeared completely. The sample was then washed by DMF (3x) and treated for 15 min with 20% piperidine in DMF. The piperidine solution was separated and combined with the DMF washes. The volume of the solution was made up to 50 ml and the extinction of the solution was read at 301 nm. The substitution of the carrier was found to be 3.1 μmol/cm^2.

Des-Gly^2-Methionine-Enkephalin

The cotton strip (20 cm) described above was shaken in a solution of TFA (25%) in DCM for 15 min, washed with DCM (2x), 10% DIEA in DCM (2x), and with DCM again (3x). The strip was shaken overnight at room temperature in 15 ml of DMF solution of 2,4,5 - trichlorophenylester of 3-(4 - fluorenylmethyloxycarbonyl - methionyl-oxymethylphenoxy) propionic acid (1) (0.2 M) and HOBt (0.2 M). The carrier substitution was determined by cleavage of the Fmoc protecting group and found to be 1 μmol/cm^2. The carrier was acetylated by means of a mixture of acetanhydride (5 ml) and pyridine (10 ml) for 30 min at room temperature, washed by DMF (3x) and DCM (3x). Three acylation cycles involving Fmoc-Phe, Fmoc-Gly and Boc-Tyr(Bu^t) were performed on the mangle as described above. The carrier was transferred to a solution of TFA (50%) and DMS (5%) in DCM. After 2 h, the carrier was washed by the same solution, eluates were combined, concentrated to a small volume and precipitated by ether. Product was dried, dissolved in 1 M AcOH, filtered and lyophilized. The crude material (30 mg) was purified by HPLC (Vydac C18, 250 x 10 mm). Product (8.6 mg, 28%), pure according to HPLC (Spherisorb ODS II, 250 x 4 mm, 75% MeOH in 0.05% TFA; k'=6.4), TLC in four systems and electrophoresis ($E^{His}_{5.7}$ 0.01, $E^{Gly}_{2.4}$ 0.73), was obtained. Amino acid analysis: Gly 1.00, Met 0.92, Tyr 0.89, Phe 0.96. Mass spectroscopy: 517 (M+H$^+$). For $C_{25}H_{32}N_4O_6S \cdot CF_3COOH \cdot 2\ H_2O$ (666.7) was calculated: 48.64% C, 5.59% H, 8.40% N; found 48.87% C, 5.32% H, 8.56% N.

Methionine-Enkephalin

The synthesis was performed analogously to the previous example, with the exception that Fmoc-Gly was used twice for the coupling. Product (15.6 mg, 46%), pure according to HPLC (Spherisorb ODS II, 250 x 4 mm, 75% MeOH in 0.05% TFA; k'=5.8), TLC in four systems and electrophoresis ($E^{His}_{5.7}$ 0.01, $E^{Gly}_{2.4}$ 0.67), was obtained. Amino acid analysis: Gly 2.00, Met 0.90, Tyr 0.86, Phe 0.99. Mass spectroscopy: 574 (M+H$^+$). For $C_{27}H_{35}N_5O_7S \cdot CF_3COOH \cdot 2\ H_2O$ (723.7) was calculated: 48.13% C, 5.57% H, 9.68% N; found 48.58% C, 5.38% H, 9.42% N.

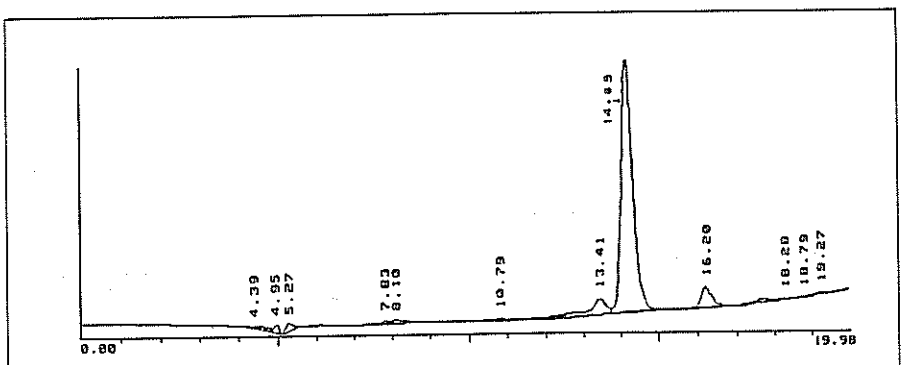

Figure 1. RP-HPLC trace of crude methionine enkephalin. Conditions: Spherisorb ODS II, 25 x 0.4 cm, 3μm, 0.5 ml/min, gradient 60-80% of MeOH in 0.05% TFA in water in 20 min, detection at 222 nm.

Methionine-Enkephalin-Modified Cellulose

A glycine-modified cellulose strip (3 x 20 cm) was acylated successively by Fmoc-Met, Fmoc-Phe, Fmoc-Gly, Fmoc-Gly and Nps-Tyr(Bu^t) in the arrangement described above. The Nps and Bu^t groups were cleaved by 7.5 M HCl in methanol (20 min). Quantitative amino acid analysis showed the enkephalin content on the carrier to be 1.6 μmol/cm^2.

Methionine-Enkephalin-Gly

The modified cellulose described in the previous example (6 cm^2) was shaken in 1 M NaOH for 1 h at room temperature. The solution was acidified by 1 M HCl to pH 3 and lyophilized. RP HPLC purification (Vydac C18, 25 x 1 cm, gradient 25-50% MeOH in 0.05% TFA in 25 min, 4 ml/min) afforded 0.95 mg (16%) of the pure product. FAB MS: 631 (M+H$^+$); amino acid analysis: Gly 3.00, Met 0.92, Tyr 0.90, Phe 0.96.

RESULTS

The experimental setup on which we performed the testing of the idea of continuous peptide synthesis was very simple and consisted of only one set of rollers and a shaker bath. Thus, all transfers were performed manually. We used a cotton strip of 3 cm width, onto which Boc-glycine was coupled by the action of dicyclohexylcarbodiimide in the presence of dimethylaminopyridine. Pretreatment of the cotton strip with trifluoroacetic acid and diisopropylethylamine prior to coupling was necessary, since the coupling of protected glycine onto the cotton is otherwise less efficient (see Reference 3). The carrier was obtained with a capacity of 3.1 μmol/cm^2 or 0.13 mmol/g. After cleavage of the Boc protecting group by trifluoroacetic acid solution, the cotton strip was treated with the 2, 4,5-trichlorophenylester of 3-(4-fluorenylmethyloxycarbonyl - methionyl - oxymethylphenoxy) propionic acid (Fmoc - Met - O - CH_2 - C_6H_4 - O$(CH_2)_3$-COOTcp) (1) and the strip

acylated successively by Fmoc-Phe, Fmoc-Gly, Fmoc-Gly and Boc-Tyr(OBu^t) in the presence of DCC and HOBt. The necessary length of time for the complete coupling was determined by bromphenol blue monitoring (7) – i.e., until the cotton strip lost its blue color. After the last step the carrier was treated with a 50% solution of trifluoroacetic acid and 5% dimethylsulfide in dichloromethane. The solution was concentrated *in vacuo*, the product was precipitated and purified by reversed phase HPLC. An RP-HPLC tracing of crude peptide is given in Figure 1. Pure methionine enkephalin, obtained in 46% yield and characterized by FAB mass spectroscopy, elemental and amino-acid analysis, was found to be fully active in the test on guinea pig ileum *in vitro*.

Des-Gly^2-methionine enkephalin was synthesized in the same manner. This analog, as one of the potential side products of the synthesis of methionine enkephalin, was chromatographically compared with the crude reaction mixture from the synthesis of methionine-enkephalin. Its presence in the reaction mixture was not detected. As expected, it was found to be biologically inactive in the guinea pig ileum test *in vitro*.

Another synthesis was performed directly on the glycine-modified cotton. This approach is useful mainly for applications in which the peptide remains covalently bound to the carrier. However, even in this case we were able to cleave the peptide from the carrier, purify and characterize it. In this case, the yield of pure peptide was much worse than in that involving the acid-labile handle. Several side products were formed during the alkaline treatment used for cleavage of the peptide from the carrier.

DISCUSSION

The basic concept of an apparatus continually producing peptides is illustrated in Figure 2. The synthesis of methionine enkephalin was chosen as an example, because we have synthesized this compound in a simulated manual version of continuous solid phase synthesis. The solid carrier, which is in the form of a tape, bears the functional group onto which the first amino acid is coupled with the use of spacers. The carrier is then led through the desired reagents and solvents with the use of a combination of rollers. Individual amino acids are sequentially coupled to the moving carrier, and in the last compartment the peptide is cleaved from the carrier. The carrier with the regenerated functional group can be directed to the beginning of the operation, and the whole synthesis may be, in the case of suitable carrier and spacer, repeated again. An advantage of this method in comparison to the classical arrangement of the solid phase synthesis in which particular steps must be performed in time sequence, is the possibility of performing all synthetic steps simultaneously. An additional advantage of this method is the fact that the product is obtained continuously and not in batches.

A diagram of one segment of the

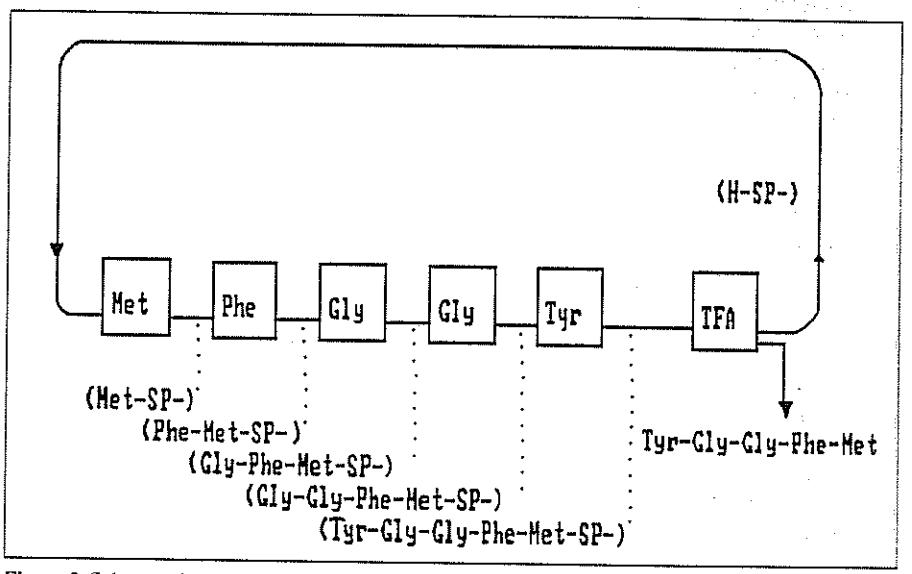

Figure 2. Scheme of the continuous synthesis of methionine enkephalin. SP, spacer group attached to cotton. The structure of growing peptide connected to the particular space of the cotton strip is given, in parenthesis.

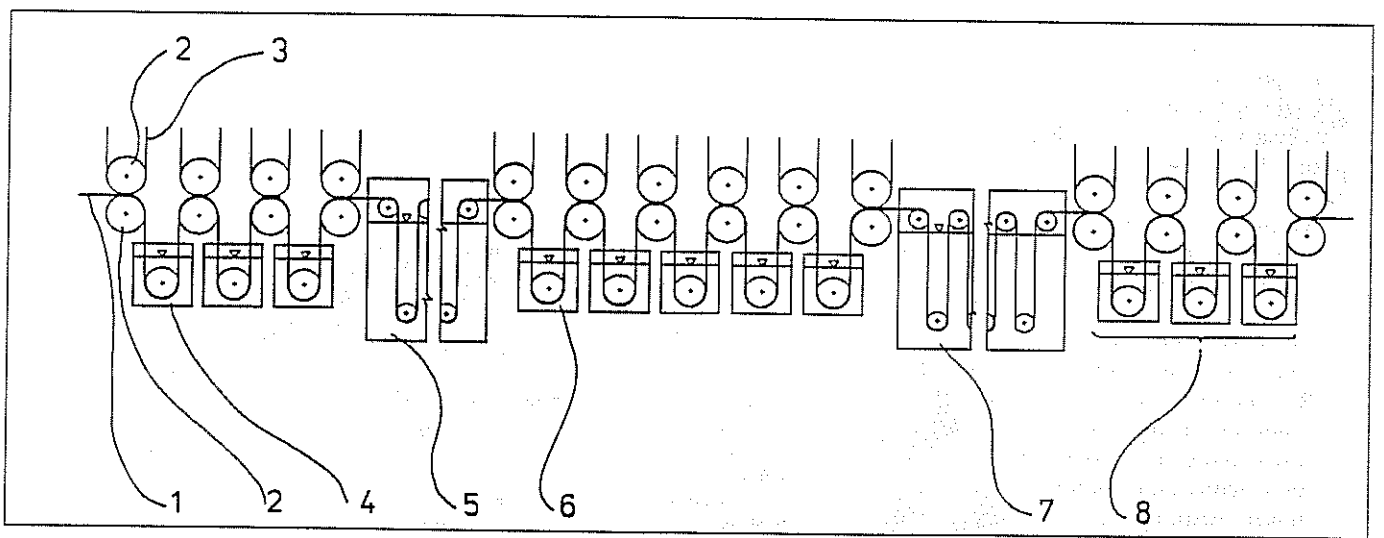

Figure 3. Scheme of the synthetic compartment in which an amino acid is coupled to the growing peptide chain. For description, see text.

synthesizer is given in Figure 3. The carrier (1) coming from a previous synthetic step is compressed between cylinders (2), thereby removing redundant liquid carried from a previous wash. Removal of liquid is facilitated by simultaneous introduction of porous material (paper, textile) (3) between the cylinders. The carrier then goes to the washing bath (4), followed again by compression between cylinders. After several repetitions of this operation, the carrier is introduced into a solution of the reagent in which cleavage of the α-amino protecting group occurs (5). The period of exposure to the deblocking bath is determined by the trajectory through which the carrier in the bath must pass. The prolongation of it is achieved by a system of loops in the bath. The carrier then leaves the deprotecting bath and undergoes washing in a series of washing solutions (6). Subsequently, the carrier passes through a solution containing the activated protected amino acid (7). The period of exposure to the activated species is determined by the time needed for completion of the coupling reaction. If we use bromphenol blue (7) to monitor the coupling reaction, it may be added, for example, to the last washing solution: and we can control the progress of the coupling visually, since the strip must be decolorized before reaching the end of this compartment. In order to speed up the coupling reaction, it is possible to use an ultrasound bath or to move the carrier over a heated cylinder. From the coupling bath, the carrier enters the system of cylinders and baths (8) again, and after that it is ready to enter another cycle in which the next amino acid is connected to the growing peptide chain. This cycle is performed in another segment which is organized analogously to the one just described. In principle it is possible to use the same baths for washing and cleavage of protecting groups and to use separate vessels only for the coupling reactions.

The system can be used for both of the most important strategies of solid phase peptide synthesis, i.e., for the Boc and Fmoc strategies. Nevertheless, synthesis using an alkali-labile α-amino-protecting group (Fmoc) allows for a significantly simpler arrangement (fewer washing steps are needed and the presently available acidolytically cleavable spacers can be used). Taking into account the nature of the carrier, it is also possible to use other protecting groups. We have shown, for example, that for synthesis on the relatively polar cotton tape, α-amino protection by the o-nitrobenzenesulfenyl group is useful, in contrast to the difficulties encountered in using this group in combination with non-polar polystyrene carriers.

The described arrangement should be useful especially for the industrial production of peptides, where the desired peptide is produced continually in an amount per time unit given by the capacity and dimensions of the carrier. Continuous synthesis of peptides can also be adapted for the small-scale synthesis of a large number of different peptides. In this case, the carrier in the form of a string would be passed through baths containing solutions of activated amino-acids, and after the end of the synthesis the whole carrier would be isolated. This would allow the synthesis of a great number of peptides of different length, the number being limited only by the capacity of the particular baths.

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