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Binary Synthesis of Multicomponent Peptide Mixtures by the Portioning–mixing Technique

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Abstract: By introducing a new operation (non-coupling), our portioning–mixing method has become suitable for preparing binary peptide libraries. We demonstrate that all the expected components of a simple library are present in the mixture. The number of components in such libraries, the molar ratio of peptides as well as the possibilities of screening are discussed.

Keywords: Binary peptide libraries; identification of peptide components; non-coupling operation; omission library; portioning–mixing synthesis

There are two independent and radically different methods that can be used for the chemical synthesis of peptide libraries: our portioning–mixing (PM) technique [1–3] first published in 1988 and the light-directed parallel chemical synthesis of Fodor *et al.* [4] introduced in 1991.

Our method ensures a dramatic improvement in the efficiency of the synthetic work. For example, an equimolar mixture of more than 3 million pentapeptides can be synthesized by executing only 100 coupling cycles [5]. The high efficiency of the method can be attributed to three groups of operations introduced to replace the coupling cycles of the solid phase method of Merrifield [6]: (i) dividing the support into equal portions; (ii) coupling a different amino acid to each portion; and (iii) mixing the portions.

The other powerful method introduced by Fodor *et al.* [4] uses an experimentally quite different approach. Spatially localized arrays of peptides were synthesized on glass microscope slides by using photolithography and photolabile protecting groups. They also described a synthetic strategy, the binary synthesis, which at first sight appears to be even more efficient than our method, since following this strategy, at least in principle, more than 4 million peptides could be synthesized by only 22 coupling cycles.

The purpose of this paper is to show that our PM technique is as suitable for carrying out binary synthesis as the photolithographic method. Only one new operation needs to be added to the three mentioned above: non-coupling [7]. Applicability of the binary libraries is also discussed.

Consideration

In order to realize binary synthesis with our PM method the cycle of operations were modified as follows:

(i) Dividing the resin into two equal portions;
(ii) Coupling one amino acid to one of the portions;

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Table 1 Components of the Binary Mixture after the Consecutive Coupling Steps

Cycle	Components
0	A
Lys	A KA
Phe	A KA FA FKA
Glu	A KA FA FKA EA EKA EFA EFKA
Leu	A KA FA FKA EA EKA EFA EFKA (uncoupled part) LA LKA LFA LFKA LEA LEKA LEFA LEFKA (coupled part)

(iii) Doing nothing (i.e. doing a 'non-coupling' operation) with the second portion;
(iv) Mixing the portions.

In the following example a binary synthesis is described leading to a low number of components to allow identification of all of them. We applied Boc technology. Boc–alanine resin ester was used as a starting material which was submitted to four consecutive cycles of four operations, using four different amino acids in couplings in the following order: Lys, Phe, Glu and Leu. In order to facilitate identification of peptides formed in the synthesis, at the end of the fourth cycle the two portions were not mixed, but treated separately.

The mixture after any coupling cycle is expected to contain all the components found in the previous coupling cycle and, in addition, the same peptides elongated by one amino acid. The starting resin (zero coupling cycle) contains Ala. This and the other expected components after the cycles Lys, Phe, Glu and Leu are summarized in Table 1 (using one-letter symbols).

The two-dimensional electrophoretic maps of the uncoupled and coupled parts of the mixture obtained after the last cycle are shown in Figs. 1 and 2, respectively. On the map in Fig. 1 there are seven spots, all of which have been assigned to the members of the planned peptide mixture with the aid of a computer program [8, 9]. As can be seen, locations of the spots of different peptides coincide with the calculated ones (indicated with asterisks). The absence of the Ala spot may be attributed to solubility problems during the working up after cleavage. No additional spot is detectable. The map of the mixture of the other eight peptides (Fig. 2) shows that each of the components is present.

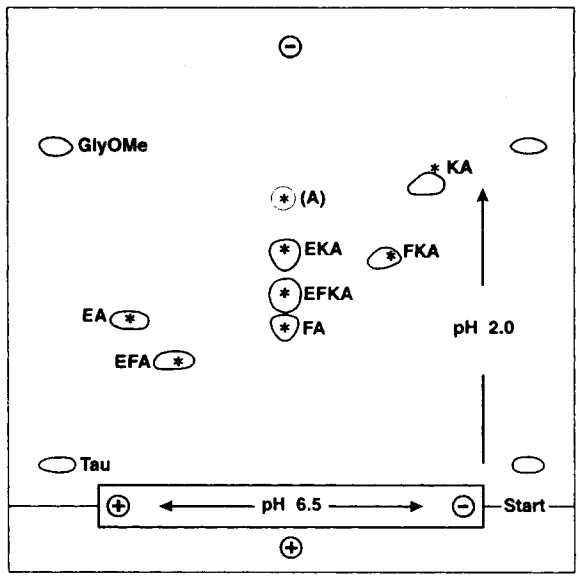

Fig. 1 Two-dimensional electrophoretic map (first run at pH 6.5; second at pH 2.0) of the uncoupled half of the peptide mixture obtained at the end of binary synthesis. Taurine and glycine methylester have been used as internal standards. The peptide sequences are written with one-letter symbols.

Number of Components and their Molar Ratios

As pointed out by Fodor *et al.* [4], the number of peptides formed in the binary synthesis (N) can be calculated from the number of coupling cycles (c) according to a simple formula: $N=2^c$. This indicates a very high efficiency since N grows exponentially with the number of coupling cycles. However, this formula only calculates the maximum number of components that are formed when every amino acid is represented only once in the series of amino acids to be coupled. In many cases this requirement cannot be maintained; for example, when the 20 L-amino acids are used as building blocks in more than 20 coupling

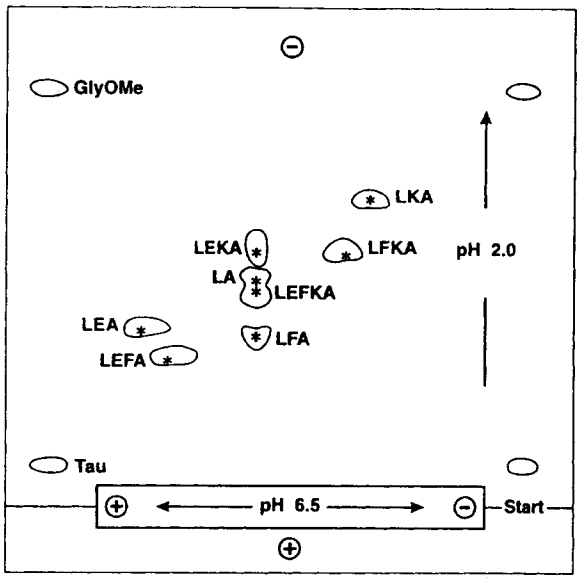

Fig. 2 Two-dimensional electrophoretic map of the other half of the peptide mixture obtained by coupling with leucine at the end of binary synthesis. (See Fig. 1.)

cycles. Multiple occurrence in the series of amino acids to be coupled has two consequences: (i) the number of components in the synthesized library is less than that calculated from the formula and (ii) the equimolarity of components is no longer maintained.

The minimum number of components is formed, for example, when the same amino acid is used in all coupling steps. In this case the components formed differ only in their length, and the total number of components is only $c + 1$ (instead of 2^c). When 10 different amino acids are used in the synthesis, 1024 components are formed. The number of components in the groups of peptides of the same lengths follow a binomial distribution and can be expressed by $\binom{c}{n}$ where n is the number of residues. When c is 10 and n varies from 0 to 10 these numbers are: 1, 10, 45, 120, 210, 252, 210, 120, 45, 10 and 1. The use of the same amino acid in all of the 10 coupling steps leads to only 11 components. In this case, of course, the binomial factors do not express the number of peptides in the groups according to length, since a single component belongs to each length. They rather express the molar ratio of the peptides in this extreme case.

Since the synthesized peptide libraries are generally submitted to screening tests, equimolarity of the components formed is an important requirement. Fortunately, one can find tricks which help to preserve equimolarity of peptides without increasing the number of coupling steps. This will be demonstrated in a simple example showing, in addition, how repetition of the use of an amino acid in the synthesis leads to the loss of equimolarity.

The expected sequences formed in the 0 to 4th cycles of a binary synthesis started with a polymer (ϕ) using A, C, D and D again in couplings are given in Table 2.

It can be seen that DCA, DC, DA and D are formed in both the 3rd and 4th cycles so their molar ratio to the other components of the final mixture is expected to be 2:1. In order to avoid duplicating of these components, the mixture coming out from the 3rd coupling cycle (DCA, DC, DA, and D; mixture II) as well as the uncoupled part (CA, C, A and ϕ, mixture I) was divided into two equal parts. One half of mixture I was extracted without coupling. The other half was mixed with one half of mixture II, then the combined mixture was submitted to the 4th coupling cycle and was finally mixed with the non-coupled part of mixture I. The components formed in the last two coupling cycles are indicated in the last two rows of Table 2. It can be seen that there is no duplication. In this example, half of mixture II remains unused. This can also be avoided by proper portioning.

Table 2 Expected Sequences using A, C, D and D again in Couplings (the Newly Formed Sequences are Written with Bold Characters)

0	ϕ
1	**A** ϕ
2	**CA C** A ϕ
3	**DCA DC DA D** CA C A ϕ
4	**DDCA DDC DDA DD DCA DC DA D** DCA DC DA D CA C A ϕ
3′	**DCA DC DA D** CA C A ϕ
4′	**DDCA DDC DDA DD DCA DC DA D** CA C A ϕ

Applicability and Screening

As has already been pointed out [5], binary synthesis is not a good choice for the systematic searching of peptide sequences since the partial libraries produced by this method cannot be completed to full libraries. If, however, one wants to explore whether or not deletions in the sequence of a longer peptide lead to active fragment(s), the method may prove to be an excellent choice. If, say, deletions in the 11–20 region of a 30-residue peptide is studied, the synthesis goes as follows: (i) a conventional solid phase synthesis is executed with the first 10 amino acids; (ii) followed by binary synthesis with the second 10 of them; and (iii) finally the synthesis is completed with 10 steps of conventional synthesis. In these 30 coupling steps, 1024 peptides are formed (instead of one), comprising all derivatives produced by all possible deletions in the 11–20 region (including the intact 30 residue and the shortest 20-residue peptide). Libraries formed when the region of deletions comprises the C-terminal or N-terminal part of the parent molecule can also easily be synthesized and the region of the deletions may also extend to the whole sequence of the molecule.

Peptide libraries are usually synthesized in order to find biologically active peptides among their components. Therefore it is important to discuss possibilities of screening. Our PM method enables the synthesis of both support-bound and free libraries. It is characteristic of our method that only one kind of peptide is formed on one bead of support (since no bead can be present in two portions in a coupling process), and as a consequence, the method described by Lam *et al.* [10] can be used in screening of support-bound binary libraries.

A quite different approach is needed when dealing with free libraries. Since binary libraries do not have a sub-library structure, our domino strategy [11] cannot be applied. One can, however, prepare partial libraries omitting one amino acid in the synthesis. These 'omission libraries' are expected to help to determine the sequence of the active component since the loss of activity (shown by the complete library) indicates that the omitted amino acid is important for the activity, i.e., it is present in the active peptide. Preservation of the activity means, on the other hand, that the omitted amino acid is not present in the molecule of the active peptide. Through the synthesis and testing of all possible omission libraries, the amino acid composition of the shortest active components can be determined. By arranging these constituent amino acids in the coupling order, the sequence can be deduced.

EXPERIMENTAL

Boc–Ala, Boc–Glu(OBzl), Boc–Leu, Boc(*Z*)–Lys and Boc–Phe were purchased from Bachem, Bubendorf, Switzerland. Dichloromethane, diisopropyl carbodiimide, diisopropyl ethylamine, dimethyl formamide, 1-hydroxybenzotriazole, trifluoroacetic acid and trifluoromethanesulphonic acid were products of Fluka AG, Buchs, Switzerland. Chloromethylated styrene–divinyl benzene (99 : 1) resin was obtained from Bio-Rad, Richmond, California (Bio-Beads SX1, 200–400 mesh, 1.26 mequiv. Cl/g). Boc–Ala–resin ester (1.0 mmol Ala/g) was prepared according to the method of Horiki *et al.* [12].

Boc–Ala resin ester weighing 100 mg was submitted to the first coupling cycle; another 100 mg of the same resin remained uncoupled. A coupling protocol described by Gutte and Merrifield [13] was adapted with slight modifications:

(i) diisopropyl carbodiimide [14] and 1-hydroxybenzotriazole [15] were used;
(ii) Boc–amino acids were added in fourfold molar excess;
(iii) the activated Boc–amino acid derivatives were dissolved in dichloromethane–dimethyl formamide mixture (3 : 1, v/v);
(iv) the coupling time was 3 h.

Mixing of the coupled and uncoupled resin samples was carried out by shaking them in a mixture of dichloromethane and dimethyl formamide (2 : 1, v/v) (11 ml) for 10 min. The suspension of the mixed resin was divided into two equal parts by pipetting (5–5 ml), the remainder (ca. 1 ml) was diluted by the same solvent mixture (10 ml), shaken for 10 min, and 5 ml volumes of the thin suspension were pipetted into both thicker slurries.

Cleavage of peptides from the resin was made by the trifluoromethanesulphonic acid technique [16, 17]. Working up of the solution of the free peptides and two-dimensional paper electrophoresis were carried out according to Furka *et al.* [3].

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