Rapid In Vitro Synthesis of Pico-mole Quantities of Peptides

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A rapid small-scale peptide synthesis method was developed based on in vitro translation system. It enables us to obtain peptides within a day. Comparing the biological activity of in vitro synthesized peptides with that of chemically synthesized ones, the activity of both peptides was confirmed to be equivalent. The recovered peptides were assumed correct in their sequence through MS analysis and estimated to be more than pmoles/run. Moreover, this method could produce highly hydrophobic peptide, otherwise unavailable.

Recent advances in instrumentation and methodology have incessantly reduced the amount of peptides/proteins required for various analyses. In particular, improvements in mass spectrometry have enabled the analysis of subfemtomole quantities of peptides/proteins. Fluorescence-based technologies usually require subpicomole amounts of peptides/proteins.[1] Though proteins have been in vivo or even in vitro synthesized,[2] peptides have been synthesized chemically and the cost is often unaffordably high. In addition, the scale of peptide synthesis is often too large in comparison to the amount required, leading to waste of resources. Furthermore, a large diversity of peptides is often required for screening with each required in a small amount.[3–5] Therefore, a method to rapidly prepare a small amount of peptides at minimal cost would be of great value having a wide range of applications. In this paper we report a standardized convenient method for the in vitro synthesis of pico-mole quantities of peptides for usual analytical purposes.

The DNA construct used in this study was made to have a tag sequence and a recognition sequence for cleaving off the aimed peptide flanking region (Figure S1).[6] Peptides were generated via mRNAs using an in vitro transcription/translation system kit PURESYSTEM® (Post Genome Inst., Tokyo, Japan) in a 50 μL of solution beginning with 5 pmole template DNA. Then, a 10 μL of streptavidin-coated magnetic beads (MAGNOTEX SA®, TaKaRa, Kyoto, Japan) were added to the reaction mixture and incubated at 37 °C for 1 h with mixing. After incubation, magnetic beads were removed using a magnet and washed with 200 μL of 0.01% BSA solution. The beads were suspended in 40 μL of Factor Xa reaction mixture (2 units of Factor Xa (QIAGEN, Valencia, USA), 20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 1 mM $CaCl_2$) and incubated at 37 °C for 1 h with stirring. The beads were then removed using a magnet to recover the supernatant. The pH of the solution was then adjusted by adding 0.4 μL of 1 M Tris-HCl (pH 8.0) buffer. A 10 μL aliquot of Factor Xa Removal resin (QIAGEN) was washed with 1 × Xa buffer (pH 8.0) and then combined with the recovered solution. The resulting suspension was incubated at room temperature for 10 min with stirring. After centrifugation, the supernatant was recovered. Peptides were purified and recovered using a Bio-Spin column. We estimated the recovery of peptides to be approximately 10 pmole in 40 μL from cathepsin E inhibitory activity assay. In the case of cDNA display-derived peptide which is attached with its encoding DNA/RNA hybrid (see Figure S1), DNase treatment was carried out to remove the non-peptide portion: 10 μL of cDNA display samples (ca. 1 pmole) was incubated for 1 h at 37 °C plus 1U of DNase I (Promega, Madison, USA).

Cathepsin E (CE) inhibitory peptides found by in vitro evolution[3] were synthesized following the protocol described above. In order to confirm the amount and activity of peptides thus obtained, these peptides were subjected to the CE inhibition activity test. On the other hand, the same peptides were prepared by conventional chemical synthesis and CE-inhibitory activities of the same sequence were also examined (Table 1). The peptides were pre-incubated with cathepsin E (CE) at a 1:1 molar ratio of enzyme (CE) to peptide dissolved in 90 μL of assay buffer. Next, 10 μL of 0.1 mM fluorogenic CE substrate was added to the solution, and then incubated at 40 °C for 10 min. The fluorescence intensity of the reaction product was measured using a fluorescence micro-plate reader.[6] The percent inhibition was calculated according to the following equation:

$$\%\text{inhibition} = 100 \times (1 - F/F_0)$$

where F and F_0 are the fluorescence intensities of the solutions containing the fluorogenic substrate and the enzyme together with or without potentially inhibitory peptide, respectively.

Based on the CE inhibitory activities of both peptides obtained by the in vitro synthesis and the chemical synthesis, a correlation curve was generated (Figure 1). Since the amount of peptide generated by in vitro synthesis is too small to allow accurate quantification, the inhibition activity values given in Table 1 are approximate based on a supposition that a similar amount of product can be obtained by the same procedure. Nevertheless, the activities thus obtained were in good correspondence with those obtained from synthesized peptides, of which amounts are accurately known, providing a correlation coefficient of 0.88 (Figure 1). This result indicates that active peptide sequences are generated and a relatively constant amount of peptides could be obtained by in vitro translation since the estimated amounts were consistent with the amounts experimentally determined beginning with a known amount of peptides chemically synthesized.

MALDI-TOFMS analysis was applied to confirm the molecular identity (i.e., molecular weight) and the amount recovered, providing a supportive result both in the molecular weight (com-

Table 1. Characteristics of in vitro or chemically synthesized peptides having a CE-inhibitory activity

Peptide No.	Amino acid sequence	Size (a.a.)	*M*r	H.P.[a]	Inhibitory activity of peptide[b]		IC_{50} /nM
					In vitro synthesis /%	Chemical synthesis/%	
1	GGSCSSCLGG RPPTIFFRLK DYKDDDDK	28	3060.4	−24.7	▲24.2[c]	▲20.7	240
2	GCGGLLPFGG QPPNPLF	17	1602.9	−20.2	▲31.5[c]	▲21.4	840
3	YCGGLLPLGG RPPTIFFRLK DYKDDDDK	28	3124.6	−20.1	▲29.8[c]	▲25.4	380
4	DGCCIIINGG RPPTIFFRLK DYKDDDDK	28	3213.7	−19.2	▲31.3[c]	▲27.1	1200
5	NYKDSCIG	8	899	−6.4	▲33.6	▲31.1	680
6	VYKDGG	6	619.7	−5.3	▲19.8	▲12.8	48000
7	SGLLFRPEGR	10	1113.3	−5.3	▲22.8	▲20.6	320
8	IPGRIGCIGG GPGGGPGGNY KDSCIG	26	2342	−3.7	▲46.7	▲42.7	—
9	NDDKIIIIPT IFGG	14	1497.7	7.8	▲27.4	▲39.9	260
10	NYKDSCIGGG GGGGGGGIT DSIIIISWIG	30	2173.3	9.5	▲60.8	▲52.8	—
11	GGRPIIIIGG	10	934.1	10.3	▲22.3	▲14.5	46000
12	NYKDSCIGGD LTPSSCGGII IISCIA	26	2617.4	17.3	▲68.9	▲59.5	—
13	NDDKIIIICC II	12	1283.5	17.6	▲37.8	▲43.9	280
14	ITDSIIIISW IG	12	1259.6	19.9	▲42.8	—[d]	—
15	IIIISCIG	8	813.06	23.8	▲24.3	▲34.9	240
16	SCGGIIIISC IA	12	1149.4	26.9	▲54.6	▲45.2	220
17	IIIIIQLIF SAW	13	1267.5	34.7	▲41.1	—[d]	—
18	NYKDIIIIPT ILIIIISCIG	20	2339.9	35.1	▲47.8	—[d]	—
19	IIIIIIIII DYKDSCIG	18	2031.6	38.6	▲42.0	—[d]	—
20	IIIISCIGII IILLPPDYKD SCIGGG	26	2682.3	39.0	▲35.1	—[e]	—

[a]Hydrophobicity. [b]The peptide amounts used for this assay were estimated for in vitro synthesis while those for chemical synthesis were quantitative (0.04 μM each). [c]These were obtained by another in vitro synthesis method through in vitro virus procedures (Details will be published elsewhere (Ref. 3)). [d]Tried but unable to synthesize. [e]Synthesis was not attended. IC_{50} represents 50% inhibitory concentration of a peptide. The triangle (▲) indicates the decrease of CE-activity relative to control (i.e., without inhibition).

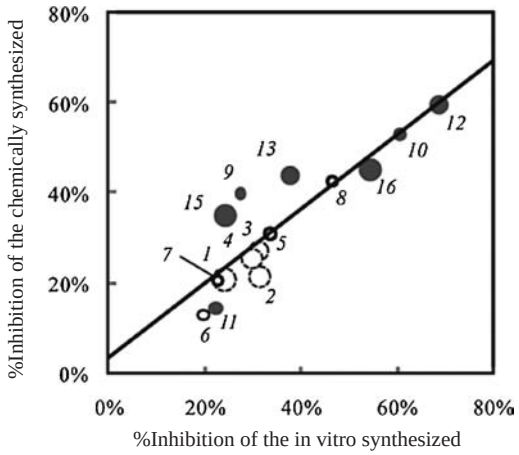

Figure 1. Correlation of inhibitory activities between the in vitro synthesized peptide and the chemically synthesized peptide. The numbers and the size of a circle represent the peptide no. and the degree of hydrophobicity in Table 1 (the negative values in an open circle while the positive ones in a filled one), respectively. As a reference, peptides obtained by cDNA display are also drawn (dotted circles).

plete match) and in the amount of product (around 10 pmoles).[6] However, MS analysis revealed the production of immature peptides (≈90% in this test case, although it seems too high in the impurity judging from the estimation based on the activities above mentioned, requiring forthcoming additional data to solve this discrepancy). Therefore, this method should be considered to be effective for all-or-none type experiments unless it can be checked by a well-established activity test as done here or by a quantitative method such as MALDI-TOFMS.

A paramount advantage of this in vitro synthetic approach is the ability to generate any kind of sequence. For example, we were able to synthesize all the peptides listed in Table 1, whereas the chemically synthetic method failed for some of the hydrophobic peptides as listed. This should be a merit of peptide synthesis at low concentrations, which is soluble for most peptides. Low cost, solubility, and rapid preparation of peptides are irreplaceable advantages of this approach. Moreover, recent advances in mass spectrometry and fluorescence-based technologies are making this kind of approach more and more effective since they require only subpicomole quantities of peptide.

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