The Influence of Mixing Conditions of Reaction Medium on Protease-Catalyzed Peptide Synthesis: The Use of Sonication

Koji TADASA, Yoshihiro YAMAMOTO, Ichizo SHIMODA and Hiroshi KAYAHARA

Laboratory of Biological Chemistry, Faculty of Agriculture, Shinshu University

Summary

Sonication was demonstrated to be a useful method mixing reactoin medium. A model reaction, Boc-Gly-OH + H-Phe-$N_2H_2Ph \rightarrow$ Boc-Gly-phe-N_2H_2Ph, was adopted to compare two mixing methods of medium, sonication and magnetic-stirring. Under biphasic system including 25% of organic solvent, sonication was preferable to magnetic-stirring for several cosolvents and showed being able to have some potentiality as mixing method. On the other hand another model reaction, Boc-Tyr (Bzl)-OH + H-Gly-$N_2H_2Ph \rightarrow$Boc-Tyr (Bzl)-Gly-N_2H_2Ph, which had not been achieved by other investigators, was progressed by the new mixing method. This new method may form other certain reaction-field in the medium.

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Protease-catalyzed peptide synthesis has attracted many workers for recent several decades due to the inherent advantages it posesses over chemically based peptide bond synthesis,[1,2] A lot of reports have been published for several years focussing upon the application of proteases to organic synthesis. Addition of organic solvent into the reaction medium affects positively a yield of product concentration. Thus biphasic system is now a very important method. Klibanov *et al.* has given the theoretical treatment for the biphasic system.[3-5]

Generally, there are two approaches to perform peptide synthesis by enzymes: kinetically controlled synthesis and equilibrium-controlled synthesis.[6-10] The biphasic system, consisting of water and water-immiscible solvent, is involved in the latter. In the latter case cosolvent serves to extract resulting products, removing them from equilibrium system. The rate of extraction might be relevant to mode of mixing water with cosolvent. Magnetic-stirring is almost accepted as a normal method of mixing reaction medium. In this paper the authors adopted sonication as a mixing method. We used a comm-

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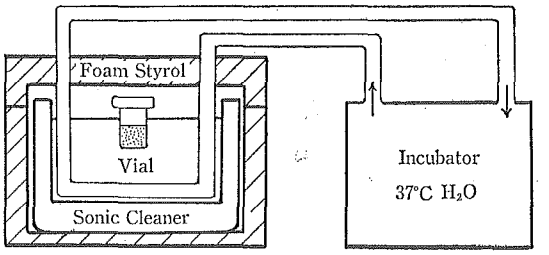

Fig. 1 Reaction system.

ercial sonic cleaner in a manner as shown in Fig 1.

Kullmann *et al.* synthesized opioid peptide, enkephalin enzymatically.[11,12] A model compound, referring to their result, was adopted as synthesis of dipeptide: Boc-Gly-OH+H-Phe-$N_2H_2Ph \cdot CF_3COOH$ and Boc (or Z)-Tyr (Bzl)-OH+H-Gly-$N_2H_2Ph \cdot CF_3COOH$. They used α-chymotrypsin and papain as catalyst. We, however, used only papain because of just clarifying influenec of sonication on the yield of the synthetic product.

Materials and Methods

Materials Boc-amino acid and Z-amino acid were prepared by a normal chemical method for synthesis excepting for Boc-Tyr (Bzl)-OH, which was purchased from Kokusan Chem. Co., Ltd. Amino acid phenylhydrazides were synthesized enzymatically (papain) from Boc-amino acid and phenylhydrazine by the method referring to Milne and Carpenter's.[13] Basic components were prepared from Boc-protected amino acid phenylhydrazide by CF_3COOH treatment. They were H-Phe-$N_2H_2Ph \cdot CF_3COOH$ and so on. The commercial sonic cleaner used was the product of Kaijo Electric Co., Ltd. (38KHz, 100W)

Methods The reaction was achieved by a preparative scale, total volume of 1m*l*. Content in one mili-liter is: 75mM of each substrate (acidic component and basic component, respectively), 0.48mM of papain (30,000USP/mg, MERCK), 82mM of cystein, 0.75m*l* of 3M acetic acid-sodium acetate buffer solution, pH 4.8, and 0.25m*l* of organic solvent (25%, v/v). The reaction was achieved at 37°C for 6-24 hrs.

Analysis After the reaction the mixture was evaporated under *vacuo* and the leftover was dissolved in one m*l* of methanol. It was used as a sample for HLPC analysis (50mm or 100mm Unisil NQ C18 column, 60% (v/v) methanol dissolved 0.1% perchloric acid, 0.5 or 1.0m*l*/min: Shimadzu LC-6A). The condensation product was evaluated by identification of its elution time with that of a compound chemically sythesized as a standard.

Results and Discussion

The organic solvent used were petroleum ether, cyclohexane, carbon tetrachloride, toluene, benzene, methylene chloride, chloroform, diethylether, ethylacetate, methanol, dimethylformamide and 1, 4-butanediol. The results of the reaction of Boc-Gly-OH with H-Phe-N_2H_2Ph are shown in Table 1. In this

Table 1 Yield of Boc-Gly-Phe-H_2H_2Ph

Cosolvent	Yield (%)	
	Sonication	Stirring
Petroleum ether	62.0	12.4
Cyclohexane	55.3	47.9
Carbon tetrachloride	61.1	58.5
Toluene	33.7	65.6
Benzene	66.7	46.8
Methylene chloride	50.9	53.8
Chloroform	42.6	60.1
Diethylether	89.0	71.2
Ethylacetate	63.4	59.7
Methanol	26.4	25.6
Dimethylformamide	5.3	8.9
1, 4-butanediol	0	31.1
*	68.0	64.9

* Without cosolvent

reaction system effect of addition of organic solvent was not shown so positively. Without cosolvent the yield by sonication is slightly larger than that by magnetic-stirring. That the effect of various cosolvent on yield is different each other, is clear by many studies. The results in Table 1 show this fact. Thus advantage of use of sonication in biphasic system is not necessarily shown to be potent for all cosolvent used, however, the effect of sonication on the product yield is very likely upon some cosolvents. Some of used cosolvents demonstrates that sonication is preferable to magnetic-stirring as mixing method.

Fig 2 shows time-course data of product. Under used conditions the maximum yield was obtained at around 12 hr-incubation.

Kullmann failed the synthsis of Boc-Tyr (Bzl)-Gly-N_2H_2Ph from Boc-Tyr (Bzl)-OH with H-Gly-N_2H_2Ph by α-chymotrypsin or papain-catalyzed reaction.[12] He obtained Boc-Tyr(Bzl)-Gly-N_2H_2Ph by benzylation of Boc-Tyr-Gly-N_2H_2Ph,

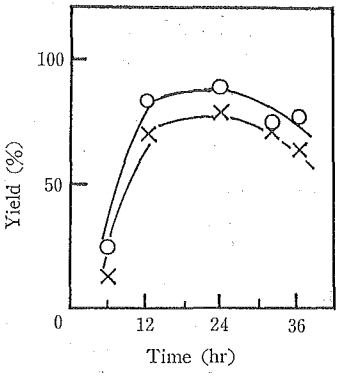

Fig. 2 Time-course of Boc-Gly-Phe-N_2H_2Ph yield. Cosolvent: Diethylether (25%, v/v)
○ : Sonication
× : Magnetic stirring

Table 2 Yield of Boc (or Z)-Tyr (Bzl)-Gly-N_2H_2Ph

Cosolvent	Yield (%)	
	Boc-	Z-
Benzene	7.6	20.6
Methylene chloride	5.5	14.2
Diethylether	5.5	29.9
Ethylacetate	13.5	3.4
*	13.3	2.7

* Without cosolvent

after the peptidation of Boc-Tyr-OH and H-Gly-N_2H_2Ph. We tried the reaction between Boc-Tyr(Bzl)-OH and H-Gly-N_2H_2Ph by sonication in several biphasic systems and succeeded to obtain Boc-Tyr(Bzl)-Gly-N_2H_2Ph, though they were not in high yield, as shown in Table 2. Further investigation will be made on this comparison, including the result by magnetic-stirring.

Judging from our results, sonication as a mixing method can be potent for peptide synthesis by protease catalysis and can be effective to form a certain field for shifting reaction not to cleavage but synthesis. It will not be sure to say the above effect positively, and we are now under further investigation.

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Abbreviation

Boc, t-butyloxycarbonyl
Bzl, benzyl
Z, benzyloxycarbonyl
Ph, phenyl

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プロテアーゼ触媒によるペプチド合成へおよぼす攪拌条件の影響：超音波の利用

只左弘治・山元義博・下田一三・茅原　紘
信州大学農学部　生物制御化学講座

プロテアーゼ触媒によるペプチド合成において，合成率に影響を与える因子として，反応系を攪拌する方法もその一つであると考えられる。我々は反応系の攪拌手段として，市販の超音波洗浄機を用いてその影響を調べた。モデル反応として，エンケファリンに含まれるジペプチド成分，Gly-Phe を得るための Boc-Gly-OH と H-Phe-N_2H_2Ph からの Boc-Gly-Phe-N_2H_2Ph への合成反応を用いた。

反応系は二相系とし，種々の有機溶媒（25%含有）のもと，超音波による攪拌と通常のマグネチックスターラーによる攪拌での合成収率を比較した。その結果，ある種の有機溶媒では超音波による攪拌のほうが，マグネチックスターラーによる攪拌より反応収率が高くなった。実験結果から，超音波による攪拌は，有機溶媒の種類と含有率によっても変化するが，反応収率に影響を与えると判断できる。また，エンケファリンの酵素合成において，Kullmann によって反応が認められないとされていた Boc-Tyr (Bzl)-OH＋H-Gly-N_2H_2Ph→Boc-Tyr (Bzl)-Gly-N_2H_2Ph 反応は，超音波法によって進行することが認められた。このような結果からも超音波による攪拌は，マグネチックスターラーによる攪拌とは異なった“反応場”を形成する可能性を示唆した。