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Hexafluoroisopropyl Alcohol is a Useful Cosolvent with Dimethylformamide for Tryptic Synthesis of Peptides

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A low-water-content solvent system, 4% H_2O in hexafluoroisopropyl alcohol/dimethylformamide (1:1, v/v), was discovered for the efficient peptide synthesis catalyzed by trypsin. This mixed solvent retains potential superiority to the Klibanov's solvent, 1% H_2O and 9% formamide in *t*-amyl alcohol, in the synthesis of longer biologically active peptides by enzymatic condensation.

Peptide synthesis catalyzed by the reverse reaction of proteases has been extensively studied on a variety of model oligopeptides.[1] The advantages of this method include that the reaction proceeds mildly and selectively. However, the wide application of proteases to the synthesis of biologically active longer peptides is restricted by some problems, *i. e.*, the achievement of high yield, the secondary hydrolysis of the products, the stability of proteases in organic media, and the solubility of long peptide fragments in the reaction media. Some objectives and approaches for the solution of these problems as summarized in Table 1 should be pointed out. Each objective is not independent, for example, when water content in the reaction media is decreased to improve the yield, proteases are often inactivated, and *vice versa*. Moreover, solving the solubility problem is indispensable as same as others for the synthesis of longer peptides.

Klibanov's group[2] recently proposed a new idea to replace water with hydrogen-bonding small molecules to achieve the very low-water-content media. The fascinating solvent mixture was *t*-amyl alcohol containing 9% formamide as a water-mimic and 1% water. They demonstrated that this solvent system successfully prevented the secondary hydrolysis in the fragment condensation of model peptides by thermolysin. However, it is still doubtful that their solvent system can be applied for the practical enzymatic synthesis of

Table 1. Objectives and approaches for protease-mediated synthesis of longer peptides

Objective	Approach
1. Higher condensation yield	Lower water content
2. Avoidance of secondary hydrolysis of product	Use of trypsin with very narrow specificity
3. Dissolution of partially protected oligopeptide segments	Polar organic solvent with capacity of hydrogen bonding
4. Retention of catalytic activity of enzyme	Stabilization of enzyme by mild solvation like water

biologically active peptides because of the increasing insolubility of longer protected segments. *t*-Amyl alcohol does not seem to be a good organic solvent during the general peptide synthesis.

The fluorinated solvents, trifluoroethanol (TFE) and hexafluoroisopropyl alcohol (HFIP), are known to be effective to dissolve poorly soluble protected oligopeptides. The strong solvation capacity could be attributed to their hydrogen bonding to insoluble peptide, which are cross-linked by intermolecular hydrogen bonds. Accordingly, we attempted to evaluate the possible utility of fluorinated solvents in the development of the practical protease-mediated peptide synthesis. We chose HFIP to examine, though it is more expensive, to be prepared for very poorly soluble peptides. We also chose trypsin, but not chymotrypsin, subtilisin, papain, or thermolysin as a protease to be tested, because it has very narrow specificity. Trypsin hydrolyzes the arginyl and lysyl bonds but never when they are protected at side chains. If all side chains of the basic amino acids except Arg or Lys for stitching point were protected, trypsin should effect only the condensation but not the secondary hydrolysis. Though the application of this strategy can avoid unfavorable hydrolysis, it inevitably involves the use of partially protected peptide segments.

In order to find out the solvent system which keeps trypsin stable and allows low water content, we examined various aqueous mixtures of water-miscible organic solvents using a model synthetic system in which Abz-Gly-Phe-Arg-OH and H-Leu-Nba (Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamine) were coupled by trypsin (TPCK treated, Sigma) to give Abz-Gly-Phe-Arg-Leu-Nba[3] (Fig. 1).[4] The reactions were monitored by reversed-phase HPLC and the yields were estimated from the integral values as compared with that of the authentic peptides. The results are summarized in Table 2. HFIP itself with or without large amount of water did not allow trypsin to catalyze the condensation. The aqueous dimethylformamide (DMF) is also examined in variety in water content. Fifty percent aqueous DMF which was frequently used by other researchers[1] afforded 30% yield. Under the intention to shift the equilibrium to the synthesis and achieve higher condensation yield, the water content was decreased, resulting in giving worse results. We tried to substitute HFIP for the majority of water in 50% aqueous DMF. With decreasing the amount of water, the yield of the tetrapeptide increased up to 83% at 4% H_2O in HFIP/DMF (1/1, v/v). The reaction reached to the equilibrium at 2 days. In this desired solvent, trypsin did not change its specificity or afford the secondary hydrolysis of the product. The dependence of yields to the water content was shown in Fig. 2. It shows that optimum content of water is 3-6%. The lower content might effect the inactivation of trypsin. In the condition containing more than 4% water, the equilibrium obviously shifted to the hydrolysis resulting in the poorer condensation yield. It should be emphasized that

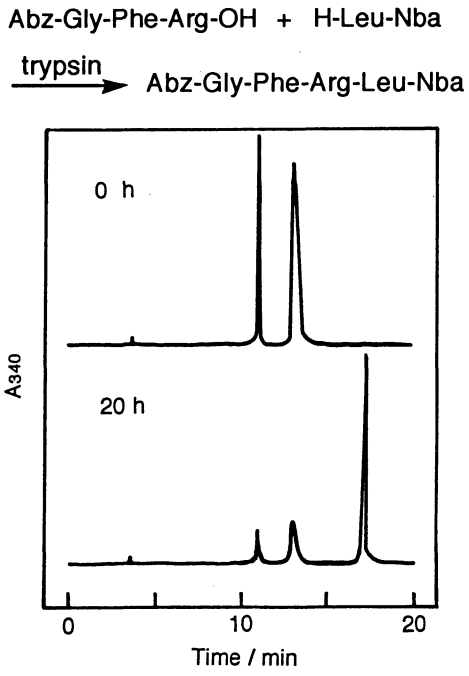

Fig. 1. The model synthetic system by tryptic condensation of the peptide. The progress was monitored by HPLC; The reaction mixture at 0 h and 20 h in 4% H_2O in HFIP/DMF (1/1, v/v). MS-GEL C18 column (4.6 mm x 150 mm), 10-70% linear gradient of CH_3CN/0.1% CF_3COOH (20 min), detected at 340 nm. Peaks correspond Abz-Gly-Phe-Arg-OH, H-Leu-Nba, and Abz-Gly-Phe-Arg-Leu-Nba in order of elution.

HFIP requires DMF as cosolvent to allow the low water content keeping trypsin active. The positive role of DMF might related to be a good acceptor to the hydrogen bonding HFIP. It is also noteworthy that significant difference in yields was not observed, when the ratio of HFIP to DMF was changed to 1/2 or 1/3. This fact indicates that the amount of HFIP is less important than the water content. In 1% H_2O/9% formamide in *t*-amyl alcohol,[2] good result was also obtained for model system in Fig. 1. However, *t*-amyl alcohol could not satisfy a requirement for the dissolution of protected peptides.[5]

Table 2. Yields of the model peptide synthesized by trypsin in various solvent systems[a]

Organic solvent	H_2O content/%	Yield/%
HFIP/DMF	50	35
(1/1, v/v)	20	44
	4	83
HFIP	50	0
	4	0
DMF	50	30
	4	0
t-Amyl alcohol with 9% formamide	1	85

a) The reaction condition is described in Ref. 4.

The effects of those low-water-content solvents on the activity of trypsin was further examined by prolonged incubation. Figure 3 shows the retention of the activity of trypsin during the incubation in buffer and various mixed organic solvents.[6] Trypsin lost its activity in 50% aqueous DMF as fast as in buffer solution probably due to the autolysis. Fifty percent aqueous HFIP quickly inactivated the enzyme suggesting that the solvent affects the three-dimensional structure of the enzyme. However, the 4% H_2O in HFIP/DMF (1/1, v/v) could retain the activity at about 40% as long as a few days at room temperature. The hydrogen bonding capacity of HFIP was accepted by DMF, probably with the formation of a stable complex, and the severity of HFIP to the conformation of the protein was tamed. It should be noted that the Klibanov's condition retained the 20% activity of trypsin.

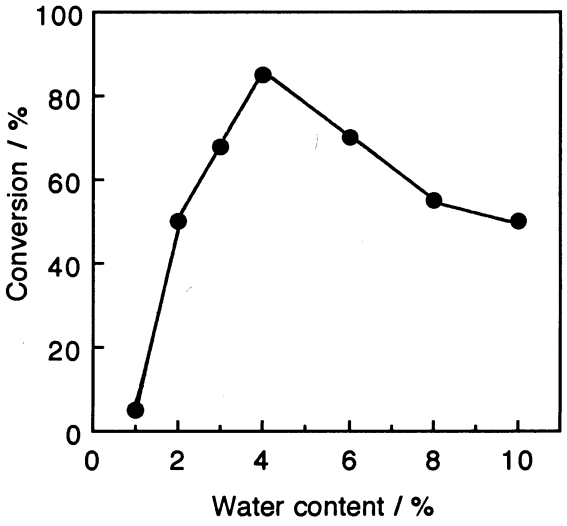

Fig. 2. Dependence of the conversion rate on the water content in HFIP/DMF (1/1, v/v).

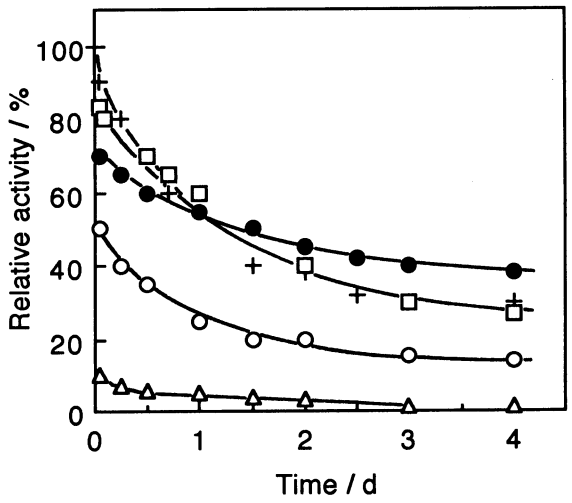

Fig. 3. Retention of the trypsin activity during the incubation in Tris·HCl buffer, pH 7.9 (+), 4% H_2O in HFIP/DMF (●), 50% aqueous DMF (□), 50% aqueous HFIP (△), 1% H_2O/9% formamide in *t*-amyl alcohol (○).

In conclusion, HFIP is a suitable solvent with DMF possessing high ability to dissolve peptide substrates and keeping the activity of trypsin with DMF as cosolvent. Furthermore, the use of HFIP allowed the water content to decrease as low as 4% or less, which was effective to shift the reaction equilibrium to the peptide-bond formation to result in a high yield.

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3) This tetrapeptide was synthesized by the solution method for an intermolecularly-quenched fluorogenic substrate for trypsin. M. J. Castillo, K. Kurachi, N. Nishino, I. Ohkubo, and J. C. Powers, *Biochemistry*, **22**, 1021 (1983).

4) To a vial containing 1.0 ml of solution of Abz-Gly-Phe-Arg-OH and H-Leu-Nba·HCl at 10 mM (1M = 1 mol·dm^{-3}) and 50 mM, respectively, in appropriate testing solvent was added Et_3N (to adjust pH 7.0) and the enzyme solution in 50 mM Tris·HCl, pH 7.9 containing 100 mM NaCl and 10 mM $CaCl_2$ (final concentration of trypsin was 40 mM). The reaction mixture was gently stirred at 25 °C. An aliquot was withdrawn for HPLC analysis with MS-GEL C18 column (4.6 mm x 150 mm) under the conditions mentioned in Fig. 1.

5) We preliminarily observed that the solvent system, 1% H_2O and 9% formamide in *t*-amyl alcohol, did not support the tryptic condensation of Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-OH and H-Trp(CHO)-Gly-Lys(ClZ)-Pro-Val-NH_2 (CHO, formyl; ClZ, 2-chlorobenzyloxycarbonyl). This failure was apparently attributed to the poor solubility of the latter segment in the mixed solvent. On the other hand, HFIP/DMF (1/1, v/v) system allowed 95% condensation. N. Nishino, M. Xu, H. Mihara, and T. Fujimoto, *Tetrahedron Lett.*, in press (1992).

6) Trypsin was incubated in various organic condensation media without peptides. An aliquot was withdrawn, appropriately diluted with aqueous buffer, and assayed for hydrolytic activity toward the corresponding substrate, Abz-Gly-Phe-Arg-Leu-Nba. From the increase in fluorescence, the percentages of the remaining activities were calculated.

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