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Immobilized *Aspergillus Oryzae* Protease Catalyzed Formation of Peptide Bonds in Organic Solvent

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Immobilized *Aspergillus oryzae* protease (AOP) catalyzed the formation of peptide bonds between *N*-protected amino acids and amino acid esters or amides in ethyl acetate. The influences of pH and reaction time on the coupling of Boc-L-Tyr and Gly-NH_2 were studied. The optimal reaction condition for this enzyme catalyzed synthesis of Boc-L-Phe-Gly-NH_2 (98.66%) was at pH 5.5 and a duration of 48 hours.

INTRODUCTION

The use of biologically active peptides for pharmaceutical purposes has increased the importance of synthesizing this compound in highly pure form. Despite the fact that chemical synthesis and recombinant DNA methods are certainly adequate techniques for most applications, neither of these methods is completely free of drawbacks. Chemical synthesis often suffers from problems such as racemization and a time-consuming side-chain protection/deprotection strategy, and the incorporation of unnatural amino acids is limited when recombinant DNA methods are used.[1] Because of the high specificity of enzymes, it is not necessary to protect the side-chain functions of the amino acid building blocks. The mildness of enzyme-catalyzed reactions also guarantees a racemization-free synthesis. Thus, the use of enzymes for peptide bond formation offers great advantages over chemical or recombinant DNA methods.[2] Enzymatic methodology has been widely used for the formation of peptide bonds.[3] Recently, a method was developed for papain catalyzed synthesis of unsymmetric cystine peptides,[4] as well as immobilized ficin catalyzed synthesis of peptides in organic solvent.[5]

Aspergillus oryzae protease was harvested from *Aspergillus oryzae* culture medium. Aspergillopeptidase B from *Aspergillus oryzae* was able to form Leu-Glu peptide bonds in aqueous solution.[6] It possesses unusual substrate specificity and is able to hydrolyze hindered and unhindered esters with high enantioselectivity.[7] Immobilized AOP also can catalyze esterification at pH 7.5 in organic solvent.[8] This ability offers an alternative to those amino acid derivatives that are not good substrates or give poor yields by using lipase or other esterase as a catalyst upon esterification. To extend this immobilized protease to other applications, we disclose the results of our investigation on the immobilized AOP catalyzed formation of peptide bonds in organic solvents.

EXPERIMENTAL SECTION

Materials

Amino acid derivatives, buffer and *Aspergillus oryzae* protease (Sigma XXIII), starch (Katayama, Japan), triethylamine (Et_3N), ethyl acetate (EtOAc) and other solvents (Merck) were purchased from the indicated suppliers.

General Methods

To a screw-cap vial 0.1 g of starch, 0.05 g of *Aspergillus oryzae* protease and 0.05 mL of 3.5 M, pH 5.5, Na_2HPO_4/citric acid buffers were added. The mixture was incubated at 37 °C with constant shaking for 30 min. The salt of amino acid ester or amide (0.2 mmole), *N*-protected amino acid (0.2 mmole) and a solution of triethylamine (0.2 mmole) in ethyl acetate (1 mL) were added to this immobilized enzyme mixture. The whole mixture was then incubated at 37 °C for 48 hours. The analysis was carried out by withdrawing a 50 μl sample from the reaction mixture and blowing it to dryness. After the addition of 1 mL of mobile phase, 10 μl of aliquot was subjected to HPLC analysis.

Quantitative Determination of Product Yields

The coupling yields were determined quantitatively using reverse-phase high performance liquid chromatogra-

Dedicated to Professor Yu-Shia Cheng on the occasion of her 65th birthday.

phy (HPLC). The HPLC was performed with a JASCO PU-980 pumping system; the absorbance at 214 nm was monitored with a JASCO UV-975 detector and recorded on a Shimadzu C-R6A chromatopac. The column (4.6 × 250 mm, Shimadzu) was packed with inertsil 5 ODS. An eluent of 40% CH_3CN in 0.26 N triethylamine-H_3PO_4 (pH 2.4) was used. The flow rate was set at 1 mL/min. The coupling yield was calculated from the peak areas with modification for the intensity factors.

Identification of Products

The products were compared with corresponding authentic compounds that were obtained by the dicyclohexylcarbodiimide method.[9] These compounds can be eluted on HPLC, developed on paper chromatography (pyridine, butanol, acetic acid and water, 24:30:6:20 v/v), or subjected to 6N HCl (110 °C, 20 h) for amino acid analysis.

RESULTS AND DISCUSSION

A suitable acyl donor and an acceptor-nucleophile are needed in the formation of a peptide bond catalyzed by *Aspergillus oryzae* protease. Therefore, we started to search randomly for a match. AOP (50 mg/mL) was immobilized with starch at pH 7.5, the same condition as esterification,[8] and was incubated with different amino acid derivatives at 37 °C in ethyl acetate. Accidentally, we found AOP catalyzed the formation of Cbz-L-Tyr-Gly-NH_2 in fair yield.

It is our purpose to seek a general method for an immobilized AOP catalyzed peptide synthesis. Therefore, the coupling of Boc-L-Tyr with an equal molar amount of Gly-NH_2 was further optimized. The results summarize in Table 1 indicate that a slightly acidic pH range at 5.5 is more suitable for the synthesis of dipeptide.

By using the same condition, we were able to synthesize a wide variety of peptides. The results are shown in Table 2. Upon coupling Cbz-L-Phe with Gly-NH_2 (Table 2, entry 8), Cbz-L-Phe-Gly-NH_2 was afforded in quantitative yield. It required at least 24 hour's reaction time to obtain a fair coupling yield, whereas the rate of synthesis is also depended on the concentration of enzyme. Immobilized AOP is quite stable and are able to reuse. However, the yields of other peptides still need to be improved. Interestingly, their substrate specificity favors Boc-L-tyrosine than Cbz-L-tyrosine is unusual and worthy to be noted (Table 2, entry 12 and 22).

The possible pathways and products in the *Aspergillus oryzae* protease catalyzed syntheses of peptide bonds are outlined in Scheme I. The enzyme may catalyze the formation of dipeptide-amides or esters (T_1), which may be subsequently hydrolyzed to form dipeptide acids (HT_1) or further elongated to produce tripeptides (ET_1). However, in the course of our investigation, tripeptide amides/esters or the dipeptide acids were not observed, this may suggest that under the conditions used, the hydrolytic activities of immobilized *Aspergillus oryzae* protease were hindered. Therefore, it showed more peptidase activity than amidase or esterase activities.

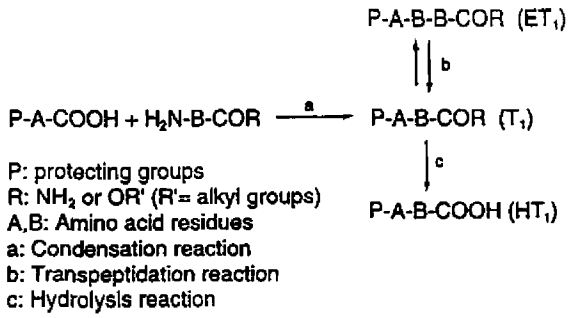
Table 1. The Effect of pH and Reaction Time on the Yield of the Reaction of *N-t*-Boc-L-Tyr with Gly-NH_2 Catalyzed by *Aspergillus oryzae* Protease[a,b]

pH\time	2 hr	4 hr	6 hr	24 hr	48 hr	72 hr
4.5	7.32	17.47	26.84	48.78	47.54	44.60
5.5	3.61	11.74	20.58	53.26	57.12	54.34
6.5	3.92	9.90	14.21	40.40	51.59	46.67
7.5	1.86	6.00	12.62	44.94	49.31	46.64
8.5	6.29	12.33	17.10	42.79	48.44	49.02

[a] *Aspergillus oryzae* protease (0.05 g) was immobilized with starch (0.1 g) in buffer (0.05 mL, 3.5 M) and shaken at 37 °C in ethyl acetate (1 mL) with Boc-L-Tyr (0.2 mmole), triethylamine (0.2 mmole) and Gly-NH_2 hydrochloride salt (0.2 mmole) for the formation of Boc-L-Tyr-Gly-NH_2.

[b] The percentage of yields were determined by HPLC analysis.

Scheme I



CONCLUSION

Since the use of protease in peptide bond formation was first reported by Bergmann and Fraenkel-Conrat,[10] a number of proteases[11-16] have been used to catalyze the formation of peptide bond. These include α-chymotrypsin, trypsin, subtilisin BPN', elastase, carboxypeptidase Y (CPD-Y), papain, pepsin and thermolysin, etc. Our results have demonstrated for the first time that *Aspergillus oryzae* protease is also capable of catalyzing the formation of peptide bonds in organic solvent. The optimal pH (same as papain)[4] for AOP catalyzed peptide synthesis is slightly differ-

Table 2. Immobilized *Aspergillus oryzae* Protease Catalyzed Formation of Dipeptides[a,b]

No.	Acyl Donor	Nucleophile	Time	Yield(%)	Product
1	Cbz-L-Asp	L-TyrOMe	48 h	13.68	Cbz-L-Asp-L-TyrOMe
2	Cbz-L-Asp	GlyOMe	48 h	--	Cbz-L-Asp-GlyOMe
3	Cbz-L-Glu	Gly-NH_2	48 h	--	Cbz-L-Glu-Gly-NH_2
4	Cbz-L-Glu	GlyOMe	9 days	1.90	Cbz-L-Glu-GlyOMe
5	Cbz-L-Glu	L-PheOMe	9 days	5.83	Cbz-L-Glu-L-PheOMe
6	Cbz-L-Glu	L-TyrOMe	48 h	22.29	Cbz-L-Glu-L-TyrOMe
7	Cbz-L-Pro	L-LeuOMe	9 days	--	Cbz-L-Pro-L-LeuOMe
8	Cbz-L-Phe	Gly-NH_2	48 h	98.66	Cbz-L-Phe-Gly-NH_2
9	Cbz-L-Phe	GlyOMe	48 h	4.80	Cbz-L-Phe-GlyOMe
10	Cbz-L-Phe	L-Pro-OBn	48 h	2.26	Cbz-L-Phe-L-Pro-OBn
11	Cbz-L-Phe	L-Leu-NH_2	48 h	--	Cbz-L-Phe-L-Leu-NH_2
12	Cbz-L-Tyr	Gly-NH_2	48 h	10.98	Cbz-L-Tyr-Gly-NH_2
13	Cbz-L-Val	L-TyrOMe	48 h	--	Cbz-L-Val-L-TyrOMe
14	Boc-L-Asn	GlyOMe	48 h	14.45	Boc-L-Asn-GlyOMe
15	Boc-L-Asn	L-Tyr-OMe	48 h	10.71	Boc-L-Asn-L-Tyr-OMe
16	Boc-L-Phe	Gly-NH_2	48 h	22.08	Boc-L-Phe-Gly-NH_2
17	Boc-L-Phe	Gly-OMe	48 h	7.17	Boc-L-Phe-Gly-OMe
18	Boc-L-Pro	Gly-NH_2	48 h	--	Boc-L-Pro-Gly-NH_2
19	Boc-L-Thr	Gly-OMe	48 h	32.21	Boc-L-Thr-Gly-OMe
20	Boc-L-Thr	L-Leu-NH_2	48 h	39.30	Boc-L-Thr-L-Leu-NH_2
21	Boc-L-Thr	L-Tyr-OMe	48 h	19.40	Boc-L-Thr-Tyr-OMe
22	Boc-L-Tyr	Gly-NH_2	48 h	57.12	Boc-L-Tyr-Gly-NH_2
23	Boc-L-Tyr	Gly-OMe	48 h	11.80	Boc-L-Tyr-Gly-OMe
24	Boc-L-Tyr	L-Leu-NH_2	48 h	7.60	Boc-L-Tyr-L-Leu-NH_2

[a] *Aspergillus oryzae* protease (0.05 g) was immobilized with starch (0.1 g) in buffer (0.05 mL, 3.5 M, pH 7.5) and shaken with indicated amino acid derivatives (0.2 mmole) at 37 °C, in ethyl acetate (1 mL).

[b] The percentage of yields were determined by HPLC analysis.

Abbreviation: Cbz: carbobenzyloxy; Me: methyl; Boc: *tert*-butyloxycarbonyl.

ent from what is optimal in hydrolysis[7] (pH 7.0) and esterification[8] (pH 7.5). This finding greatly enhanced the applicability of this enzyme. It also opens the possibility of using other proteases to catalyze the formation of peptide bonds by this immobilization method.

In addition, many bioactive peptides contain an essential C-terminal amide not affordable by existing recombinant DNA techniques as bacterial expression hosts lack the proper α-amidating enzyme(s).[17] This problem is generally overcomed by enzyme catalyzed coupling of an amino acid amide to the α-carboxylic acid of the C-terminal end on a peptide precursor.[18-19] Another way is using amino acid amides as nucleophiles to perform an enzyme catalyzed transpeptidation to exchange the last amino acid on the C-terminal end.[20] Our results provide an additional tool in achieving this aim. Further studies on the formation of other peptide bonds with amino acid amides as nucleophiles are in progress.

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Key Words

Aspergillus oryzae protease; Peptide synthesis; Immobilization; Organic solvent.

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