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Model Studies on Carboxypeptidase Y Catalyzed Peptide Synthesis in an Aqueous-Organic Two-Phase System

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Carboxypeptidase Y catalyzes in a biphasic system containing carbon tetrachloride and carbonate buffer the reaction of *Z*-Phe-O*Me* and various *Z*- and *Boc*-protected dipeptide methyl esters with Val-NH_2 and Leu-NH_2, respectively. This method has been applied to the synthesis of the corresponding N-protected tripeptide amides on a preparative scale. Using a substrate—nucleophile ratio of only 1:2 or 1:3 the peptide derivatives are obtained in yields of 56-97%.

(Keywords: Carboxypeptidase Y catalyzed peptide bond formation; Enzymic synthesis in biphasic systems; Peptide synthesis)

Modelluntersuchungen zur Carboxypeptidase Y-katalysierten Peptidsynthese im wäßrig-organischen Zweiphasensystem

Carboxypeptidase Y katalysiert in einem Zweiphasensystem aus Tetrachlormethan und Carbonatpuffer die Reaktion von *Z*-Phe-O*Me* und verschiedenen *Z*- und *Boc*-geschützten Dipeptidmethylestern mit Val-NH_2 bzw. Leu-NH_2. Nach diesem Verfahren werden die entsprechenden N-geschützen Tripeptidamide in präparativem Maßstab hergestellt. Bei einem Substrat-Nucleophil-Verhältnis von nur 1:2 oder 1:3 erhält man die Peptidderivate in Ausbeuten von 56—97%.

Abbreviations: IUPAC-IUB rules for peptides are followed, see Eur. J. Biochem. **27**, 201 (1972). *Boc* = *tert*-butyloxycarbonyl, *Z* = benzyloxycarbonyl, -O*Me* = methyl ester, HPLC = high performance liquid chromatography, TLC = thin layer chromatography, *CPD-Y* = carboxypeptidase Y.

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Introduction

At present a rapid development in the field of enzymatic peptide synthesis can be observed, because the usefulness of this method of peptide bond formation has been convincingly demonstrated in recent years. For reviews see[1–7]. Besides a lot of model reactions carried out in order to investigate structural requirements of both substrates and nucleophiles as well as experimental conditions for an enzyme catalyzed coupling reaction, synthesis of biologically active peptides has also drawn much attention. Amongst the proteases studied in this respect so far, carboxypeptidase Y from yeast has been a subject of special interest. This is underlined by several papers from *Johansen* and his co-workers who have done a great deal of basic work in exploring the utility of *CPD-Y* for enzymatic peptide synthesis[8,9]. Their recent investigations have revealed some outstanding features of *CPD-Y* which make it a useful tool for peptide bond formation. Having a wide specificity for amino acid side chains, this protease can be used in a great number of coupling reactions. In contrast to the endopeptidases, there is no risk of splitting any internal peptide bonds. *CPD-Y* shows high activity towards ester substrates at $pH > 9$, where its peptidase and amidase activity are strongly diminished. This suggests the use of amino acid and peptide esters as substrates and amino acids or amino acid amides as nucleophiles in *CPD-Y* catalyzed peptide synthesis. In the present paper we wish to report on our results of *CPD-Y* catalyzed synthesis of model peptides on a preparative scale in an aqueous-organic two-phase system.

Results and Discussion

The syntheses were carried out in a 10-ml-reaction vessel containing a buffered aqueous phase (*pH* 9.5) and 35% (*v/v*) carbon tetrachloride with a total volume of 5.7 ml. *Z*-Phe-O*Me* and the methyl esters of various *Z*- and *Boc*-protected dipeptides were used as substrates in the *CPD-Y* catalyzed reaction with Val-NH_2 and Leu-NH_2 as nucleophilic component. The reaction was started by the addition of an aqueous solution of *CPD-Y* to the vigorously stirred reaction mixture and then followed by TLC. There was no further control of the *pH* during the experiment. After the reaction had been completed, the mixture was worked up as described in the experimental part and the peptide product isolated. The results are summarized in Table 1 which clearly demonstrates that *CPD-Y* is also an effective catalyst for peptide coupling in a biphasic solvent system. The synthesized peptide models were obtained in good yields using an economic substrate-nucleophile

Table 1. *Carboxypeptidase* Y *catalyzed peptide synthesis using* 0.2 *M carbonate buffer* (*pH* 9.5) *and* 35% (*v*/*v*) CCl_4[a]

Substrate (35 m*M*)	Nucleophile[b] (m*M*)	Synthesized Peptide	Time (min)	Yield (%)
Z-Phe-O*Me*	Val-NH_2 (105)	*Z*-Phe-Val-NH_2	60	97[c]
	Val-NH_2 (70)		60	92[c]
Z-Ala-Phe-O*Me*	Leu-NH_2 (70)	*Z*-Ala-Phe-Leu-NH_2	40	76[c]
	Val-NH_2 (70)	*Z*-Ala-Phe-Val-NH_2	60	63[c]
Z-Leu-Ala-O*Me*	Leu-NH_2 (70)	*Z*-Leu-Ala-Leu-NH_2	30	95[c]
	Val-NH_2 (70)	*Z*-Leu-Ala-Val-NH_2	40	80[c]
Z-Ala-Tyr-O*Me*	Leu-NH_2 (105)	*Z*-Ala-Tyr-Leu-NH_2	120	60[c,d]
Boc-Leu-Ala-O*Me*	Val-NH_2 (70)	*Boc*-Leu-Ala-Val-NH_2	20	63
Boc-Phe-Leu-O*Me*	Val-NH_2 (70)	*Boc*-Phe-Leu-Val-NH_2	240	56
Boc-Leu-Phe-O*Me*	Val-NH_2 (70)	*Boc*-Leu-Phe-Val-NH_2	240	62

[a] 2 μM *CPD-Y*, room temperature.
[b] Used as hydrochloride.
[c] Product precipitated.
[d] Reaction carried out in suspension.

ratio of only 1:2 and 1:3, respectively. The necessary reaction times correspond to those observed in other protease catalyzed peptide coupling reactions in water-organic solvent two-phase systems[10–13].

As it can be seen from Table 1, *Boc*-protected dipeptide methyl esters are also suitable substrates for *CPD-Y* giving *Boc*-protected tripeptide amides in good yields.

While the formed Z-protected tripeptide amides precipitated during the reaction, this was not the case with the *Boc*-protected peptide amides. The somewhat lower yields of the latter may be attributed to their increased solubility in the organic phase. It seems not to be a stringent condition to have the reactants completely dissolved at the beginning of the reaction. Thus, using *Z*-Ala-Tyr-O*Me* as a substrate, the synthesis was carried out in suspension. However, to obtain a comparable good yield in this model reaction a threefold amount of Leu-NH_2 and a longer reaction time were necessary.

The physical constants of the synthesized peptide amides have been determined after recrystallization. They are summarized in Table 2.

As it can be concluded from our preliminary results, *CPD-Y* catalyzed synthesis of N-protected peptide amides is possible under mild reaction conditions in a buffered biphasic water-organic solvent

Table 2. *Physical properties of the enzymic reaction products*

Peptide	m.p. (°C)	Solvent for recrystall.[a]	Optical rotation[b] $[\alpha]_D^{22}$	HPLC[c] Capacity factor k' eluent a	eluent b
Z-Phe-Val-NH_2	241-242	A	−1.5	2.92	1.32
Z-Ala-Phe-Leu-NH_2	225-226	A	−51.0	4.65	1.70
Z-Ala-Phe-Val-NH_2	255-256	B	−15.0	—	—
Z-Leu-Ala-Leu-NH_2	242-244	A	−28.7	4.83	1.85
Z-Leu-Ala-Val-NH_2	278-280	A	−12.8	2.94	1.20
Z-Ala-Tyr-Leu-NH_2	180-185	A	−30.5	1.61	0.62
Boc-Leu-Ala-Val-NH_2	247-248	A	−19.0	—	—
Boc-Phe-Leu-Val-NH_2	206-208	C	−8.0	6.90	2.77
Boc-Leu-Phe-Val-NH_2	233-234	D	−19.0	6.74	2.70

[a] A, *Me*OH/H_2O; B, *Me*OH/$CHCl_3$; C, *EtOAc*/*Me*OH; D, *Me*OH/*EtOAc*.
[b] $c = 1$, *DMF*.
[c] See Experimental.

system without controlling the *pH* during the reaction. In comparison to previous reports[8] a more economic substrate-nucleophile ratio has been used. The procedure is also applicable to the synthesis of *Boc*-protected tripeptide amides.

Experimental

Carboxypeptidase Y in aqueous solution was a gift from the Institute of Protein Research, Academy of Sciences, Poustchino, USSR. The concentration of the enzyme was determined spectrophotometrically using $E_{280nm}^{1\%} = 14.8$[14]. Melting points were determined with a *Boëtius* apparatus and are corrected. Optical rotations (Polamat A of VEB Carl Zeiss Jena, 1 dm cells) are accurate to at least $\pm 0.5°$. TLC was performed on silica-precoated foils (Kavalier, Czechoslovakia) using the following solvent systems: $CHCl_3$/*Me*OH (9:1); $CHCl_3$/*n*-propanol (9:1); $CHCl_3$/acetone/*Me*OH (7:2:1). Elemental analyses of all synthesized compounds were within acceptable limits. For HPLC a Liquochrom 307 High Performance Liquid Chromatograph (Labor MIM, Hungaria) was used in connection with a Dukol UV-detector (Carl Zeiss, GDR) operating at 254 nm. Isocratic elution was performed by *Me*OH/*i*-propanol/dioxane/0.1% acetic acid (57 + 2.5 + 0.5 + 40) in a Hewlett-Packard pre-packed column 200 × 4.6 mm packed with Lichrosorb RP-18, 10 μm (eluent a). A home-packed 250 × 4 mm column (Si-100 based RP-18 material, 10 μm, kindly provided by Dr. *H. Engelhardt*, Saarbrücken, FRG) was used for *Me*OH/*n*-propanol/dioxane/0.1% H_3PO_4 (50 + 10 + 0.55 + 40) as eluent b. Methanol was used as t_0-marker for the calculation of the capacity factors k'.

General Procedure for Enzymatic Peptide Synthesis

A solution of the amino acid amide (0.4–0.6 mmol) in 1 ml 0.1 *M* KCl containing 1 m*M EDTA* was adjusted with a concentrated solution of NaOH to *pH* 9.5. Then 0.2 *M* carbonate buffer (*pH* 9.5) was added up to a volume of 3.3 ml. This was followed by the addition of 0.2 mmol ester compound in 2 ml carbon tetrachloride and 0.4 ml of an aqueous solution of *CPD-Y* (0.7 mg). The resulting mixture was stirred at room temperature until the ester component was no more detectable by TLC. It was then poured into 20 ml *Me*OH. After evaporation of the organic solvents in vacuo, 1 *M* HCl was added, the product collected on a glass filter, successively washed with water, saturated $NaHCO_3$ solution, water, and then dried in vacuo to constant weight. The physical data (Table 2) are those of the recrystallized products.

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