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C-Terminal Peptide Amidation Catalyzed by Orange Flavedo Peptide Amidase**

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The presence of a C-terminal α-amido group on the peptide chain is essential for the biological activity of many peptide hormones.[1] Amidated peptides are usually prepared by solid-phase synthesis on benzhydrylamine resins or by the ammonolysis of C-terminal peptide esters, which can be prepared by conventional peptide synthesis. Recombinant DNA technology allows the production of longer peptides by fermentation, but these products lack the C-terminal amido group. The combination of rDNA technology with chemical modification of the C-terminus requires the laborious protection of the functional groups in the side chains. Therefore, an enzymatic method for the introduction of an α-amido group is highly desirable.

Enzymatic amidation of peptides with ammonia as nucleophile has rarely been described.[2] Short model peptides and amino acid derivatives were amidated by protease-catalyzed ammonolysis of the corresponding esters.[3, 4] In spite of the advantages of enzymatic methods in peptide synthesis,[5] the application of proteases for peptide amidation is limited, mainly by the risk of undesired proteolysis. In 1990 we isolated an unusual enzyme which can hydrolyze peptide amides at the C terminus without cleavage of peptide bonds or amido groups in the side chain.[6] The enzyme was isolated from the flavedo of oranges and characterized as a peptide amidase.[7] The broad substrate specificity of the enzyme makes it universally applicable for the deprotection of α-carboxy groups in enzymatic peptide synthesis.[5]

Our aim was to use the peptide amidase to catalyze the reverse reaction, that is, the C-terminal amidation of peptides [Eq. (1); R^1 = amino acid residue or peptide residue; R^2 = side chain of an amino acid), which has not been described

$$R^1CONHCH(R^2)COOH + NH_3 \overset{\text{amidase}}{\rightleftharpoons} R^1CONHCH(R^2)CONH_2 + H_2O \quad (1)$$

up to now. In protease-catalyzed peptide synthesis,[5] the "kinetic approach" is generally considered more effective than the reverse of peptide hydrolysis ("thermodynamic approach").[5] Owing to the lack of esterase activity of the peptide amidase, only a thermodynamic approach was possible. In aqueous solutions the equilibrium of the amidase-catalyzed reaction lies far to the side of the hydrolysis products. Initial attempts to detect the reverse reaction with an excess of ammonium acetate in water-miscible solvents were unsuccessful.

Here we report on a more systematic study of amidation reactions with the dipeptide Z-Gly-Phe-OH (Z = benzyloxycarbonyl) as the acyl component and ammonium hydrogencarbonate as the ammonium source. When the reaction was carried out in acetonitrile in the presence of small amounts of water,[8] the peptide amide could be detected. The optimal dipeptide concentration was 0.05 M. Increasing the concentration to 0.1 M resulted in a viscous reaction mixture owing to the precipitation of the ammonium salt of the dipeptide, which excludes the substrate from the reaction. As shown in Figure 1, the optimal ratio of acyl component to ammonium hydrogencarbonate was 1:1.4. The slight excess of the ammonium salt maintains slightly alkaline conditions for

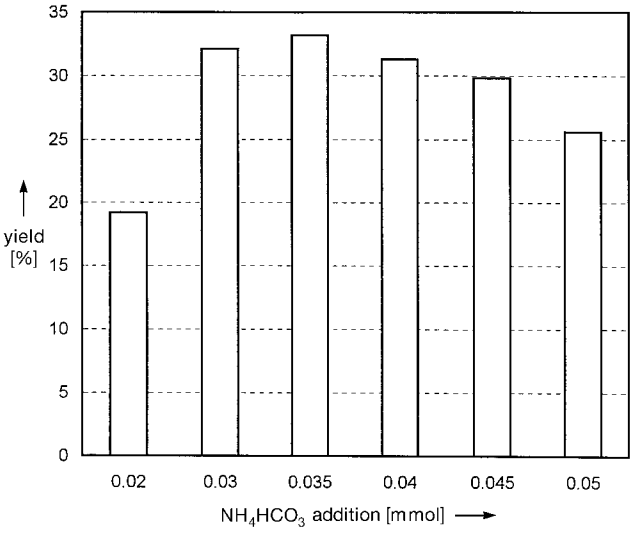

Figure 1. Influence of the ammonium concentration on the product yield in the peptide amidase catalyzed synthesis of Z-Gly-Phe-NH_2 in acetonitrile in the presence of 5% water. Conditions: 0.025 mmol of Z-Gly-Phe-OH, 2 mg of amidase, 0.5 mL total volume, 48 h, 40 °C.

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the amidase-catalyzed reaction and minimizes the risk of precipitation of ammonium salts.

With the exception of diammonium hydrogenphosphate, very low amide yields were obtained with ammonium salts of strong inorganic and organic acids as the ammonium source (Table 1). In the presence of these salts, the reaction medium

Table 1. Peptide amidase catalyzed amidation of Z-Gly-Phe-OH by various ammonium salts in acetonitrile containing 5% of water at various temperatures.[a]

Ammonium salt	T [°C]	Yield [%]
ammonium hydrogencarbonate	25	20.0
	30	26.0
	35	29.0
	40	31.5
	45	30.5
ammonium sulfate	40	0.5
diammonium hydrogenphosphate	40	32.0
ammonium acetate	40	5.0
diammonium tartrate	40	8.5
ammonium carbamate	40	4.0[b]
	40	24.5[c]
	40	31.0[d]

[a] 0.025 mmol of Z-Gly-Phe-OH, 0.04 mmol of ammonium salt, 2 mg of amidase, 0.50 mL total volume, 48 h. [b, c, d] With 0.04, 0.025, and 0.015 mmol of ammonium carbamate, respectively, as donor.

does not attain the basicity required for optimal enzyme activity,[7] as indicated by measurement of the apparent pH value. The use of a stronger base such as ammonium carbamate resulted in negligible amidation under standard conditions. The yield was considerably improved by decreasing the ammonium carbamate concentration in the reaction mixture (Table 1). Clearly, the reaction requires a slightly alkaline "pH value", which is controlled by the concentration of the nucleophile.

The dependence of the amide synthesis on the water concentration is shown in Figure 2. The best results were

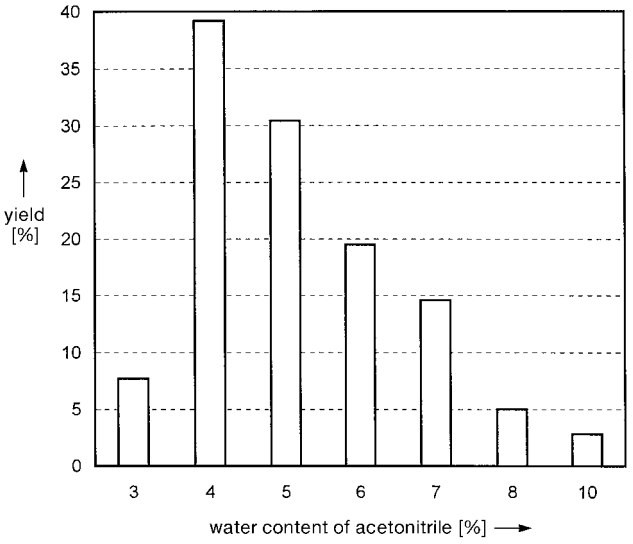

Figure 2. Influence of the water concentration on the product yield in the peptide amidase catalyzed synthesis of Z-Gly-Phe-NH_2 in acetonitrile. Conditions: 0.025 mmol of Z-Gly-Phe-OH, 0.04 mmol of NH_4HCO_3, 2 mg of amidase, 0.5 mL total volume, 72 h, 40 °C.

obtained in acetonitrile with a water content of 5%. Decreasing the water content to 4% had a positive effect on the amidation, but under these conditions the ammonium salt of the dipeptide tends to precipitate. The immediate precipitation of the ammonium salt is presumably responsible for the dramatic drop in the amide yield for a water content of 3%. No amidation was detected in other water-miscible organic solvents, such as 2-propanol, 1,4-butandiol, tetrahydrofuran, diisopropyl ether, or ethyl acetate, in the presence of 5% water.

The optimum reaction temperature was 40 °C (Table 1).

Figure 3 shows the course of the enzymatic amidation of Z-Gly-Phe-OH with time. The maximum product yield at

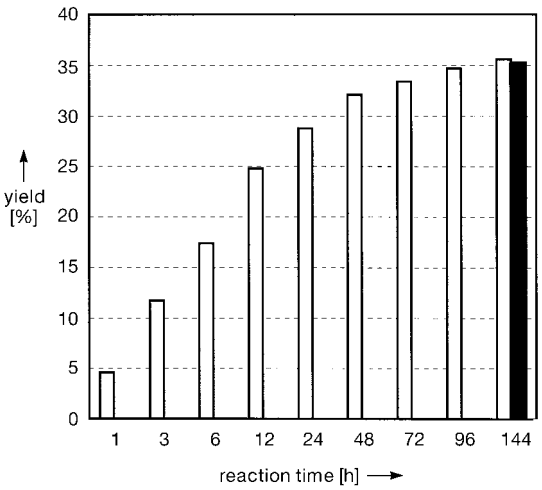

Figure 3. Course of the peptide amidase catalyzed amidation of Z-Gly-Phe-OH in acetonitrile containing 5% water with time. Conditions: 0.025 mmol of Z-Gly-Phe-OH, 0.035 mmol of NH_4HCO_3, 2 mg of amidase, 0.5 mL total volume, 40 °C. Open bars: yield of Z-Gly-Phe-NH_2; solid bars: a second portion of enzyme (2 mg) was added after 72 h of incubation.

equilibrium was reached after two days and remained nearly unchanged for a further four days. The addition of fresh enzyme after 72 h did not influence the amide yield over a further period of 72 h.

In the hydrolysis reaction, the enzyme was nonspecific with respect to the amino acid side chains of the peptide.[7] The yield of the enzymatic peptide amidation in acetonitrile seems to be dependent on the structure of the peptide substrate (Table 2). Poor yields were obtained in mixtures that led to the formation of insoluble ammonium salts. The dramatic decrease in yield for Z-Gly-Tyr-NH_2 as compared to Z-Gly-Phe-NH_2 cannot be explained at present.

The limitations of the enymatic amidation of peptides result mainly from solubility parameters. Precipitation of peptide substrates as ammonium salts under reaction conditions must be avoided. The first prerequisite for the successful application of peptide amidase for amidation is to find an optimal solvent mixture with regard to the solubility of the substrate. The excess of nucleophile required for the reaction is surprisingly low. The ammonium donor should be chosen so as to maintain a suitable pH. These requirements must be fulfilled individually for each peptide. Our results demonstrate that enzymatic amidation of unactivated α-carboxy

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Table 2. Peptide amidase catalyzed amidation of peptides in acetonitrile containing 5% of water.[a]

Peptide	Yield [%] of amide	Precipitation of ammonium salt	t_{ret} [min] amide	peptide
Z-Gly-Phe-OH	33.5	no	6.73	8.84
Z-Gly-Leu-OH	27.5	yes[b]	5.83	7.89
Z-Gly-Tyr-OH	1.0	yes	3.96	4.71
Z-Ala-Phe-OH	8.0	yes[c]	8.08	11.04
Z-Leu-Phe-OH	23.0	no	19.84	26.83
Z-Pro-Phe-OH	21.5	no	11.58	14.57
Z-Phe-Ala-OH	33.0	yes[b]	8.10	9.90
Z-Ala-Pro-Leu-OH	24.0	no	8.75	10.75

[a] 0.025 mmol of peptide, 0.035 mmol of NH_4HCO_3, 2 mg of amidase, 0.50 mL total volume, 72 h, 40 °C. [b] Precipitation after 40 h. [c] Immediate precipitation.

groups in peptides is possible. The necessary enzyme can be isolated from orange peel, a waste product of the juice industry, by a simple two-stage extraction procedure that yields 300 – 500 U per kilogram of peel.[9] Since the unchanged peptide can be easily recycled, enzymatic amidation could be economically feasable after further optimization.

Experimental Section

Typical procedure for the amidase-catalyzed amidation of peptides: The reactions were carried out in 2-mL plastic tubes with screw caps, which were incubated in a rotatory shaker at 1000 rpm and various temperatures. The benzyloxycarbonyl dipeptide (0.025 mmol) was dissolved in acetonitrile (475 μL). The required water and ammonium concentrations were adjusted by the addition of water and 2 M NH_4HCO_3 to give the final calculated volume of 500 μL. After the addition of lyophylized amidase (2 mg), the suspension was shaken at 40 °C for several days. Before the termination of the reaction, the apparent pH value of the mixture was checked with indicator paper. The reactions were terminated by diluting the mixture with 50% aqueous methanol (1.5 mL) containing 1% trifluoroacetic acid (TFA). The product yield was estimated by HPLC on an RP-18 column (Merck, WP-300, 5 μm, 25 × 0.4 cm) operated isocratically with a mixture of methanol and 0.1% TFA (45/55) at 1 mL min^{-1}. Detection was carried out at 254 nm. The newly formed peak corresponded to the peptide amide and had a retention time identical to that of a standard sample. To follow the course of the amidase-catalyzed amidation of Z-Gly-Phe-OH with time, reactions were performed in several test tubes and terminated after a given time interval.

Synthesis of Z-Gly-Phe-NH_2: Z-Gly-Phe-OH (357 mg, 1 mmol) was dissolved in a mixture of acetonitrile (19 mL) and water (0.3 mL). After a solution of NH_4HCO_3 (0.7 mL, 2 M) and amidase (80 mg) was added, the mixture was shaken in a sealed vessel at 35 °C for 4 d. According to HPLC analysis the yield of Z-Gly-Phe-NH_2 was 31%. The neutral product was isolated by cation- and anion-exchange chromatography and crystallized from ethyl acetate/petroleum ether to give 82 mg of dipeptide amide (23% yield, pure according to TLC and HPLC analysis). The m.p. of 133 – 134 °C corresponds to that of standard sample. Amino acid analysis: Gly 1.00, Phe 0.99; FAB-MS: 356.1 $[M+H]^+$.

The peptide amidase was isolated according to the literature procedure.[7, 9] The lyophilized enzyme had an activity of 0.2 U mg^{-1} with Z-Gly-Tyr-NH_2 as substrate. The peptides were purchased from Bachem (Germany), and ammonium salts were obtained from Fluka (Switzerland).

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Secondary Bonding between Chalcogens or Pnicogens and Halogens**

Gregory A. Landrum and Roald Hoffmann*

The more crystal structures we know, the clearer it becomes that in the solid state there are many contacts in the range between a bond and a van der Waals interaction. N. W. Alcock introduced the useful term "secondary bonding" for these, and formulated a set of rules for their occurrence and directionality.[1]

For electron-rich main-group systems there are two popular ways to address in a qualitative way the electronic structure of secondary bonded species—either as a manifestation of hypervalence (electron-rich three-center or multicenter bonding[2]) or as directional donor – acceptor bonding.[3] We feel these approaches are in fact equivalent, though we doubt that the number of energetic electrons expended on the demerits of one or the other chemical views is exhausted.

In recent work we have used the concept of donor – acceptor interactions to interpret calculations on hypervalent bonding in the trihalides and hydrogen bihalides,[4] intermolecular interactions in R_2QX_2 (Q = Se, Te; X = I, Br, Cl),[5] and the secondary bonding in dimers of Ph_2IX and XF_3 (X = I, Br, Cl).[6] These studies, and the importance of directionality in secondary bonding,[7] have led us to look more broadly at the nature of secondary interactions. In this work searches of the Cambridge Structural Database[8] (CSD) have been used to determine the prevalence and geometries of secondary bonding in crystals containing chalcogens or Group 15

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