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Single-Step Enzymatic Conversion of Peptide Amides to Esters.

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Abstract: It is shown that the C-terminal amide groups in N-protected peptides could be efficiently replaced by ester groups via kinetically-controlled papain-catalyzed acyl transfer reactions in aqueous methanol. The specificity of papain was substantially shifted towards the cleavage of C-terminal amide bond in dipeptide substrates by methanol, thus suppressing undesired peptide bond cleavage during esterification.

Enzymatic peptide synthesis possesses a number of potential advantages and has become an alternative to conventional chemical methods. As a rule, amino acid amides show high nucleophilic efficiency in the protease-catalyzed acyl transfer reactions, and are widely used as acyl acceptors in enzymatic peptide synthesis[1,2]. However, using of amino components in the form of amide generates a rather inconvenient problem of removing the amide blocking group from the C-terminus of the resulting peptide for further elongation of the peptide chain. In order to gain full advantage from the enzymatic route of peptide synthesis the conversion of C-terminus to ester or acid should be carried out enzymatically.

Kawashiro et al.[3] proposed a two-step enzymatic process for the substitution of amide groups with alkyl ester groups where the carboxypeptidase Y catalyzed deamidation of peptides[4] is followed by esterification of the C-terminal carboxylic groups by papain in aqueous ethanol. It has been shown that α-chymotrypsin and trypsin could be also used for esterification of carboxylic groups in peptides[5]. However, since the enzyme active site acylation by carboxyl group is comparably slow, high enzyme concentrations and/or long exposition times should be used for the thermodynamic esterification, which considerably enhances the formation of undesired side products due to the peptide bond cleavage.

In this paper we report on the applicability of a single step kinetically controlled enzymatic method for the replacement of C-terminal amide groups of peptide with alkyl ester groups. Bz-Arg-NH_2 was used as a model substrate for the study of papain-catalyzed acyl-transfer reactions in aqueous methanol[6]. The time-course of the papain-catalyzed reaction of Bz-Arg-NH_2 with methanol showed that up to 85% of the amide substrate was transformed into the corresponding methyl ester during 8 hours incubation at pH 8.0, 25° C and water content 1%. The experiments at different substrate concentrations (0.5, 1 and 2 mM) showed that the reaction of papain with Bz-Arg-NH_2 proceeded according to the pseudo-first-order kinetics with the rate constant equal to 3.55×10^{-3} min^{-1} $(mg/ml)^{-1}$. At the same time, the secondary hydrolysis of the ester product is slow when compared with the rate of ester synthesis from Bz-Arg-NH_2. Under the similar reaction conditions (with 3 mg/ml of papain) only 60% of the

S_3	S_2	S_1	↓	S'_1	S'_2	
Z -	Gly -	Leu	-	NH_2		METHANOL
Bz -	Ala -	Val	-	NH_2		
	Z -	Gly	-	Leu -	NH_2	WATER
	Bz -	Ala	-	Val -	NH_2	

Figure 1. Specificity of papain towards dipeptide substrates in water and methanol.↓ Denotes scissile bond. Numeration of enzyme subsites as in[10].

carboxylic group of Bz-Arg-OH was esterified by methanol. The ester yield of the latter reaction is determined by the thermodynamic equilibrium of the esterification at the given activities of water and methanol in the reaction medium. Thus, the ester yield in the papain-catalyzed reaction of Bz-Arg-NH_2 with methanol exceeded its thermodynamically determined level under the reaction conditions. Since the reaction of papain with amide substrates is faster than with the corresponding compounds with free C-terminal carboxylic groups, lower enzyme concentrations and/or shorter exposition times could be used in the case of amides. This decreases the possibility of the occurrence of undesired side reactions and peptide bond cleavage in the case of peptide substrates.

Water content in the aqueous methanol substantially affected the papain catalyzed esterification of Bz-Arg-NH_2. Maximum ester yield (85%) was achieved in the presence of 1% of water. The ester yield decreased with increasing water contentdue to theenhanced rate of acyl enzyme hydrolysis. At 15% water content up to 85% of the amide was hydrolyzed. The decrease in the ester yield at lower water content was caused by the lower reaction rate under these conditions: 17% of the substrate remained unreacted in the mixture with 0.5% water content after 12 hours.

Since the amidase and esterase activities of papain have different pH-optimum, the effect of pH on the acyl-transfer reaction was studied. The pH-optimum of the ester synthesis was in the range 6.6 to 7.6 which corresponds to the pH-optimum of the amidase activity of papain in water. The lower ester yields at low pH were most probably caused by a decrease in the overall reaction rate rather than by changes in the relative rates of acyl-enzyme hydrolysis and alcoholysis.

Further, the reactions of two dipeptide substrates, Z-Gly-Leu-NH_2 and Bz-Ala-Val-NH_2[7], with papain in aqueous methanol were studied. In order to check wider applicability of the proposed method we chose the dipeptide substrates where the cleavage of peptide bond rather than the removal of amide group corresponds to the papain specificity in water. It is essential for papain reactions that the substrate has hydrophobic substituents in P_2 and P'_1 positions[8,9] (see Fig.1). When the reactions were carried out in water, papain hydrolyzed the Gly-Leu and Val-Ala bonds in the above substrates in accordance with its specificity along with negligible amide group removal. Nevertheless, in aqueous methanol containing 2% of water as much as 87% of the reacted Bz-Ala-Val-NH_2 was converted to the desired ester product (Table). With Z-Gly-Leu-NH_2 as substrate the ester yield (24%) was considerably lower. Thus, the yields of ester formation in the papain-catalyzed reactions depended on the amino acid residue in P_1 position being lower in the case of more hydrophobic leucine. This finding is in conformity with an earlier

Table. Papain-catalyzed cleavage of dipeptide amides in aqueous methanol.

SUBSTRATE	WATER %	PRODUCTS, %				
		Z-Gly-OH	Z-Gly-Leu-OH	Z-Gly-Leu-NH_2	Z-Gly-OMe	Z-Gly-Leu-OMe
Z-Gly-Leu-NH_2	2	11	51	8	4	24
	4	19	49	4	8	20
	6	34	40	-	10	16
	20	79	8	-	13	-
		Bz-Ala-OH	Bz-Ala-Val-OH	Bz-Ala-Val-NH_2	Bz-Ala-OMe	Bz-Ala-Val-OMe
Bz-Ala-Val-NH_2	2	3	5	18	4	70
	4	6	8	4	14	68
	6	8	12	-	20	50
	2[a]	1	29	27	8	55

Reaction conditions: pH 8.5, t°=40°C, [S]=10 mM, [papain]=4 mg/ml, incubation time 24 hours.

[a]) Papain immobilized on XAD-8

observation[3] that the dipeptides containing Gly and Ala, but not Val, in P_1 position could be easily esterified by papain in aqueous ethanol.

In the case of both substrates, methanol increased substantially the selectivity of papain towards amide bond in comparison with the peptide bond. Up to 75% of the amide bonds were cleaved at 2% water content. The change in the selectivity of papain is most probably caused by the suppression of favorable hydrophobic interactions between the enzyme S'_1 subsite and potential hydrophobic P'_1 residue of the substrate at the peptide bond cleavage. Since papain recognizes hydrophobic substituents also in P'_2 and P'_3 positions[9], it could be assumed that the suppression of peptide bond cleavage by alcohol in the case of longer peptides is even more significant. It should be noted here that in a biphasic system where the water activity in the enzyme microenvironment is high, the introductions of hydrophobic amino acids into the P_2 position of dipeptide substrates lead to the peptide bond cleavage by papain instead of the esterification of C-terminus[10].

Since the immobilization of papain on XAD carrier[11] improved its properties for thermodynamic ester synthesis in aqueous ethanol[3], we also carried out an experiment with immobilized enzyme. However, as seen in Table, we did not observed ester yield enhancement in the kinetically controlled process.

It could be concluded that the papain-catalyzed alcoholysis of peptide amides in aqueous alcohol is more efficient for obtaining peptides with alkyl ester group in the C-terminus than thermodynamically controlled esterification proposed in[3]. Due to the wide substrate specificity of papain and efficient suppression of its peptidase activity by alcohols, this enzymatic method may be widely applicable for the esterification of longer peptides with various amino acid compositions.

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6. Papain (EC 3.4.22.2) solutions (50mg/ml) were made in 1 M citrate/phosphate buffer containing 10 mM dithiotreitol and 100 mM EDTA. Reactions were performed in 1 ml polypropylene tubes. Into the tubes containing methanol solutions of the substrate and nucleophile microliter quantities of the enzyme solution were added. The tubes were rapidly shaken and kept in thermostat on an overhead stirrer. Aliquots of the reaction mixture and analyzed by HPLC using a Series 8800 Gradient System (Du Pont Instruments, USA). A 4.6x250 mm Silasorb C_{18} column was used. Water/methanol mixture containing 0.1% of trifluoroacetic acid was used as an eluent. The substrate and products were detected at 255 nm.
7. The substrates were synthesized enzymatically according to the method in Ref.2
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9. Papain (50 mg) was dissolved in phosphate/citrate buffer (4.5 ml, pH 7.5) and stirred with 200 mg XAD-8 Amberlite during 2 hours. The solution was filtered through a glass filter, 3ml of buffer containing 100 mM dithiotreitol and 100 mM EDTA was added to the enzyme. After 1 hour incubation the solution was removed and the enzyme was dried on the glass filter. Aliquots of XAD-papain were weighted into the reaction tubes.
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