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The Application of Papain, Ficin and Clostripain in Kinetically Controlled Peptide Synthesis in Frozen Aqueous Solutions

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Abstract: The capability of the cysteine proteases ficin, papain and clostripain to form peptide bonds in frozen aqueous solutions was investigated. Freezing the reaction mixture resulted in increased peptide yields in kinetically controlled coupling of Bz–Arg–OEt with various amino acid amides and dipeptides. Under these conditions, peptide yields increased up to 70% depending on the enzyme and the amino component used. Enzyme-catalysed peptide syntheses were carried out under optimized reaction conditions (temperature, amino component concentration and pH before freezing) using the condensation of Bz–Arg–OEt and H–Leu–NH_2 as a model reaction.

Keywords: Peptide synthesis; frozen aqueous solution; ficin; papain; clostripain

Abbreviations

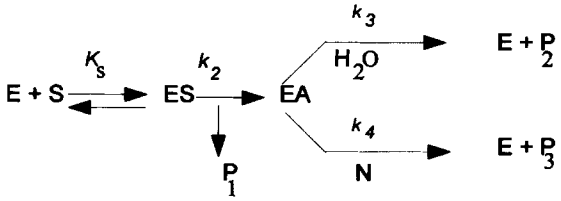
ACN, acetonitrile; Bz, benzoyl-; DTT, dithioerythritol; Mal, maleyl-.

INTRODUCTION

Freezing the reaction mixture can significantly increase peptide yield in protease-catalysed peptide synthesis [1–9]. According to the 'freeze–concentration model' [10, 11] the yield-increasing effect results from concentration of the reactants in the unfrozen liquid phase which is in equilibrium with the solid solvent phase. Consequently, aminolysis of the acyl enzyme is favoured over hydrolysis under kinetically controlled conditions (Scheme 1).

Owing to acyl enzyme intermediate formation and their broad specificity, cysteine proteases are suitable catalysts for kinetically controlled peptide synthesis [12–17]. Nevertheless, only limited information about their application to peptide synthesis in ice is available. Recently, we proved the catalytic effect of ficin (EC 3.4.22.3) on the formation of peptide bonds in frozen aqueous solutions [9]. In ice, papain (EC 3.4.22.2) has only been used to catalyse the condensation of Bz–Arg–OEt with the amides of histidine, leucine and valine and various unprotected amino acids [8] and in the synthesis of Mal-Phe-Ala-Ala-Ala-OH [1].

The application of the cysteine protease clostri-



Scheme 1 Protease-catalysed kinetically controlled peptide synthesis. E, enzyme; S, acyl donor ester; ES, Michaelis complex; EA, acyl enzyme; P_1, acyl donor ester leaving group; P_2, hydrolysis product; P_3, peptide product; N, nucleophile (amino component).

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pain (EC 3.3.22.8) to peptide synthesis is of particular interest attributed to its restricted specificity for Arg–X–bonds [18, 19] including proline at the P_1' position (S′ subsite nomenclature according to [20]). Although clostripain-catalysed acyl transfer reactions were carried out in aqueous solution [21, 22], the enzyme has not yet been used in ice to form peptide bonds.

In this study, we compare the catalytic effect of clostripain, papain and ficin on the condensation of Bz–Arg–OEt with various dipeptides and amino acid amides in ice. Clostripain- and papain-catalysed syntheses of Bz–Arg-Leu–NH_2 were carried out at various temperatures, pHs and nucleophile concentrations in order to optimize the reaction conditions. Ficin-catalysed peptide synthesis in frozen aqueous solution has been optimized previously using the same model reaction [9].

MATERIALS AND METHODS

Chemicals

Ficin from *Ficus carica latex* (Serva, Germany, 0.56 U/mg, determined titrimetrically at pH 6.5, Bz–Arg–OEt as substrate) and papain from *Carica papaya* (Merck, Germany, 15.5 U/mg, determined titrimetrically at pH 8.0, Bz–Arg–OEt as substrate) were used without further purification. Clostripain from *Clostridium histolyticum* (80 U/ml, determined spectrophotometrically at pH 7.6, Bz–Arg–OEt as substrate) was purified as described in [23]. Bz–Arg–OEt, amino acid amides and dipeptides were obtained from Bachem, Germany.

Enzyme-catalysed Peptide Synthesis

Peptide synthesis reactions were performed in 1 ml polypropylene tubes in total volumes of 0.1 ml (−15 °C) and 1 ml (25 °C), respectively. The ester substrate was dissolved in water, amino components in 70 mM KCl/0.7 mM EDTA. After adjustion to the desired pH and prior enzyme addition solutions were cooled to 0 °C. Enzyme solutions (papain in 5 mM DTT, ficin in water, clostripain after activation for 3 h in 1 mM $CaCl_2$/5 mM DTT) were added, the tubes rapidly shaken and inserted into liquid nitrogen for 30 s and transferred into a cryostate (Haake, Germany).

Reactions were stopped by the addition of 100 µl TFA (2.5%, v/v). Control reactions were performed at 25 °C as described without freezing.

The ester concentration was 2 mM, and enzyme concentrations were chosen to achieve complete ester conversion within at most 24 h (ficin, 0.1–10 mg/ml; papain, 0.1–0.4 mg/ml; clostripain, 0.08–32 µg/ml). Maximum peptide yields were obtained after reaction times varying according to the nucleophile used from 1 to 4 h at 25 °C and from 2 to 24 h at −15 °C.

HPLC Analysis

HPLC analysis was carried out using a Shimadzu HPLC system (SCL 10-A system controller, LC 10-AS liquid chromatograph, SCL-10-A autoinjector with sample cooling system, SPD-10-AV UV detector and a Compaq personal computer with Shimadzu LC-10 software). An RP-18 Lichrosorb column (250 × 4 mm, Merck, Germany) was used.

Mixtures of ACN and TFA (0.1%, v/v) served as eluents running isocratic and gradient elution, respectively. The substrate and product concentrations were detected at 254 nm. Since the aminolysis and the hydrolysis products contain the same chromophore, the absorption coefficients were assumed to be equal.

RESULTS AND DISCUSSION

Increased peptide yields at temperatures below 0 °C in papain-catalysed synthesis of Bz-Arg-Leu-NH_2 (Figure 1) can be explained by the 'freeze–concentration model' based on the assumption that ice crystal formation is accompanied by concentration of the reactants in the liquid phase resulting in favoured aminolysis of the acyl enzyme [10, 11]. At −20 °C reaction occurred more slowly and the ester substrate was not completely converted within the fixed reaction time. In papain-catalysed coupling reactions the acyl enzyme seems to be saturated at a nucleophile concentration of 20 mM (50% as free base) because at higher amino component concentrations only a slight increase in peptide yield was observed (Figure 2). Especially at low nucleophile concentrations the advantage of freezing the reaction mixture becomes evident.

In clostripain-catalysed synthesis of Bz–Arg-Leu–NH_2 a similar dependence of peptide yield on temperature and amino component concentration was observed (results not shown).

Above pH 7.8, the expected positive influence of the amount of nucleophilic free base could not be observed in papain-catalysed synthesis of Bz–Arg-Leu-NH_2 (Figure 3). In ice the decrease in peptide

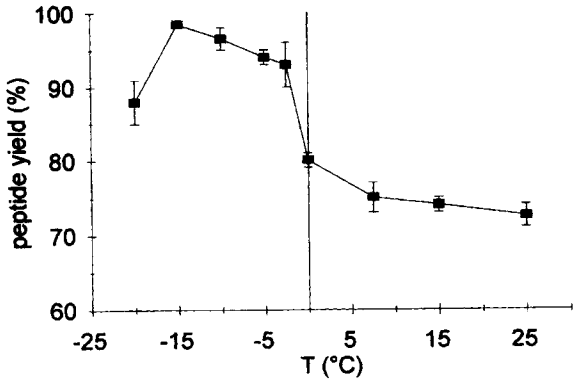

Figure 1 Dependence of peptide yield on temperature in papain-catalysed synthesis of Bz-Arg-Leu-NH_2: 2 mM Bz-Arg-OEt, 50 mM H-Leu-NH_2, pH 7.8.

yield above pH 7.8 is based on a slower ester conversion as described by Groeger et al. [15], whereas at room temperature increased ester hydrolysis was also observed. This corresponds to observations of Schellenberger *et al.* [24] who reported decreased efficiency of papain-catalysed acyl transfer at higher pH.

Non-enzymatic ester hydrolysis which does not take place in ice can also contribute to lower peptide yields at room temperature.

Contrary to papain, in clostripain-catalysed synthesis there is a marked difference between the pH dependence in ice and at room temperature (Figure 4). The decreasing yield above the pH optimum in ice as well as at room temperature is attributed to the same effects as discussed for papain.

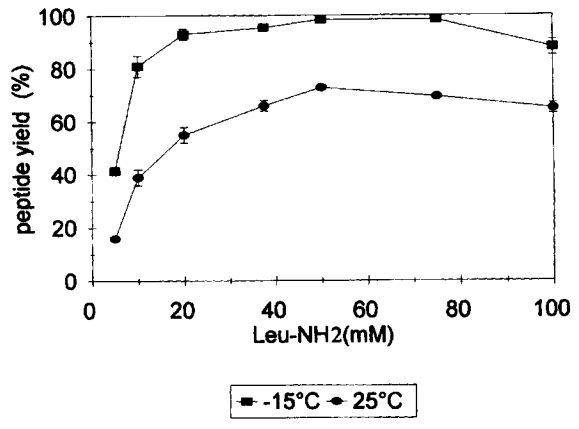

Figure 2 Dependence of peptide yield on amino component concentration in papain-catalysed synthesis of Bz-Arg-Leu-NH_2: 2 mM Bz-Arg-OEt, pH 7.8.

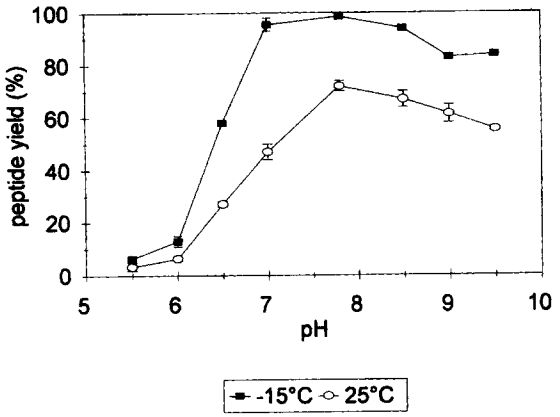

Figure 3 Dependence of peptide yield on pH in papain-catalysed synthesis of Bz-Arg-Leu-NH_2: 2 mM Bz-Arg-OEt, 50 mM H-Leu-NH_2.

According to these results and the optimized reaction conditions of the ficin-catalysed synthesis of Bz-Arg-Leu-NH_2 reported previously [9], ficin- and papain-catalysed condensations of Bz-Arg-OEt and various amino acid amides and dipeptides were performed at −15 °C and at pH 7.8. Clostripain-catalysed reactions were carried out at pH 7.0 and at the same temperature. The concentrations of the amino components were varied according to their pK_a to give a free base concentration of 10 mM.

As shown in Table 1, the peptide yields obtained in ficin-, papain- and clostripain-catalysed condensations of Bz-Arg-OEt with various amino components clearly demonstrate the positive influence of freezing the reaction mixture, whereas the quantitative effect of freezing is different according to the enzyme and

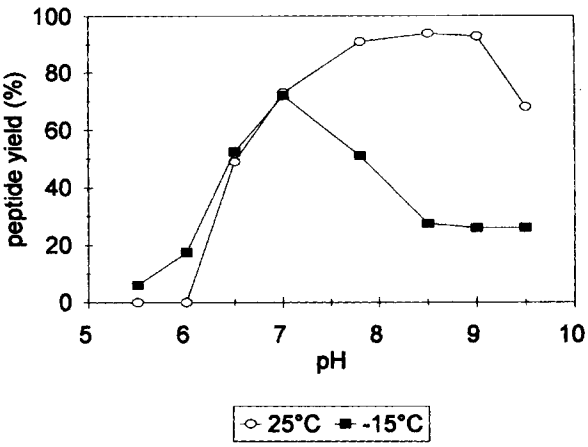

Figure 4 Dependence of peptide yield on pH in clostripain-catalysed synthesis of Bz-Arg-Leu-NH_2: 2 mM Bz-Arg-OEt, 50 mM H-Leu-NH_2.

the nucleophile used. In ice, secondary hydrolysis of the peptide product did not take place as long as acyl donor ester was present in the reaction mixture. Using H–Arg–NH_2 as amino component, a by-product was formed as well as the dipeptide amide. This side reaction was suppressed in ice, too. At −15 °C only 2–4% of by-product was found, depending on the enzyme used, while at 25 °C 9–15% was obtained.

The results we obtained in papain-catalysed syntheses at room temperature are in agreement with the observations of Schuster *et al.* [25] that acylpapain shows a preferred binding of hydrophobic residues. Positively charged amino components such as Arg–NH_2 are better bound than nucleophiles with a negative charge such as Asp–NH_2 or the dipeptides we used. In ice, these differences were markedly less distinct. The lack of binding of Pro–NH_2 or of D-Leu–NH_2 at room temperature could not be overcome in ice, indicating the steric requirements of the S′-subsite.

Concerning this fact, the behaviour of the cysteine proteases ficin and papain is different from that of the serine protease α-chymotrypsin, showing remarkably higher yields in coupling of nucleophiles with D-configuration in ice compared with the inefficient coupling at room temperature [1, 26].

In general, we observed a yield-increasing effect of freezing whereas Littlemore et al. [8] reported both an increase and a decrease comparing peptide yields of papain-catalysed reactions in ice and at room temperature. This different result is probably caused by the different reaction conditions used.

Using ficin as the biocatalyst, for most amino components tested the increase of peptide yield in ice was less than that observed in papain-catalysed reactions. In clostripain-catalysed syntheses, good coupling yields obtained at room temperature (except using nucleophiles with Asp- and Pro- residues in P_1' and P_2' positions which gave substantially lower yields) could only be improved slightly by freezing. The results obtained with H–D-Leu–NH_2 as nucleophile are in good agreement with the observations of Ullmann and Jakubke [22] who reported that clostripain does not require the P_1' position to have the L-configuration.

CONCLUSIONS

Papain proved to be an outstandingly suitable catalyst in ice and offers new possibilities in coupling inefficient nucleophilic amino components in high yields. As demonstrated in clostripain-catalysed coupling, freezing the reaction mixture is not generally advantageous in kinetically controlled peptide synthesis catalysed by cysteine proteases.

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Dedicated to Professor Harald Tschesche on the occasion of his 60th birthday.

Table 1 Protease-catalysed Condensation of Bz–Arg–OEt with Amino Acid Amides and Dipeptides

Nucleophile	Peptide yield (%)					
	Ficin		Papain		Clostripain	
	−15 °C	25 °C	−15 °C	25 °C	−15 °C	25 °C
H–Ala–NH_2	49	24	84	31	83	74
H–Arg–NH_2	52	18	97	36	83	72
H–Asp–NH_2	36	6	46	1	14	8
H–Gly–NH_2	81	26	93	20	94	87
H–Leu–NH_2	89	30	93	56	91	83
H–D-Leu–NH_2	0	0	0	0	79	65
H–Pro–NH_2	0	0	0	0	32	27
H–Ala-Ala–OH	50	33	91	26	73	55
H–Ala-Asp–OH	23	11	47	10	13	11
H–Ala-Leu–OH	71	3	68	8	74	56
H–Ala-Pro–OH	35	12	78	9	42	40

2 mM Bz–Arg–OEt, 10 mM effective nucleophile concentration, ficin, papain, pH 7.8; clostripain, pH 7.0.

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