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REVIEW ARTICLE

Nonconventional Protease Catalysis in Frozen Aqueous Solutions

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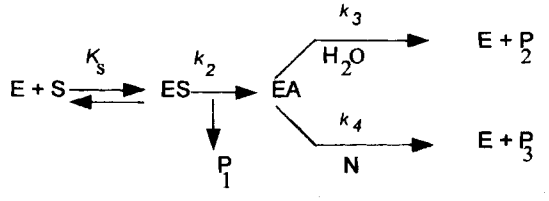
During the past decade proteases have been widely used as catalysts in peptide synthesis. Unfortunately, they are not ideal ligases. Enzymatic peptide synthesis in frozen aqueous systems has been developed as an approach towards the suppression of competitive reactions. This paper summarizes reports concerning the behaviour of non-enzymatic as well as of enzyme-catalysed reactions when the reaction mixture is frozen. The advantages of freezing the reaction mixture in serine and cysteine protease-catalysed peptide synthesis, the influence of modified reaction conditions and the possible reasons for the yield-increasing effect of freezing are discussed.

Keywords: peptide synthesis; frozen aqueous solution; protease

INTRODUCTION

The application of enzymes for catalysing peptide bond synthesis provides an attractive addition to the chemical-synthetic and recombinant techniques in various situations. Because of their regio- and stereospecificity they guarantee racemization-free segment condensation and only minimal protection of side-chain functions is required. The ideal enzyme to catalyse peptide bond formation should be the ribosomal peptidyltransferase which exhibits its full catalytic function independently of the side-chain functions of the amino acids to be ligated. Since this enzyme requires coordination with numerous ribosomal factors it is not suitable for a simple enzymatic-technical application. Microbial non-ribosomal multi-enzyme complexes which are involved in the synthesis of peptide antibiotics do not possess a general applicability in peptide synthesis. Therefore, the only alternative to native peptide ligases are proteases, due to the principle of microscopic reversibility. In the mid-1970s it was established that proteases can be used as catalysts for peptide bond formation on a preparative scale [1]. The development of two distinguishable strategies in enzymatic peptide synthesis is the subject of several recent reviews [1–4]. The direct reversal of peptide hydrolysis (equilibrium-controlled approach) ends in an equilibrium shifted to the thermodynamically more stable cleavage products. In the kinetic approach, serine or cysteine proteases catalyse the transfer of



Scheme 1 Protease-catalysed kinetically controlled peptide synthesis. E, enzyme; S, acyl donor ester; ES, Michaelis complex; EA, acyl enzyme; P_1, acyl donor leaving group; P_2, hydrolysis product; P_3, peptide product; N, amino component (from [5]).

Abbreviations: Mal-, maleyl-; -OEt(Cl), monochloroethyl ester.

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donor acyl groups to nucleophilic acceptor molecules to give a transient accumulation of the peptide product far above the equilibrium concentration (Scheme 1).

Unfortunately, proteases cannot act as ideal ligases. In the kinetic approach, efficiency of peptide bond formation is affected by competitive hydrolysis of the acyl enzyme. Other peptide yield-limiting factors are proteolysis of the peptide product as well as the acyl donor in segment condensation.

These undesired side reactions can be suppressed using chemically modified enzymes, enzyme mutants or zymogens as catalysts and by medium engineering or manipulation of the reaction conditions in the kinetic approach [2]. Using a highly specific leaving group of the acyl donor results in increased peptide yield in serine and cysteine protease-catalysed peptide synthesis [6, 7]. Competitive hydrolysis of the acyl enzyme can be suppressed by addition of organic solvents to the aqueous reaction mixture or by the use of micro-aqueous monophasic organic solvents [2]. In contrast, the concentration of water can also be reduced by freezing aqueous reaction systems. Lineweaver reported as early as in 1939 that freezing decreases the rate of proteolysis [8]. Therefore, the possibility arises of suppressing hydrolytic side reactions in enzyme-catalysed peptide synthesis by freezing the reaction mixture.

INFLUENCE OF FREEZING ON NON-ENZYMATIC AND ENZYME-CATALYSED REACTIONS

Non-enzymatic Reactions

In 1961 Grant *et al.* [9] described an unusual lability of the β-lactam bond of penicillin in frozen aqueous systems. They observed imidazole- and base-catalysed cleavage of the β-lactam at temperatures between − 5 and − 30 °C in frozen state but not in supercooled systems and attributed this effect to a favourable substrate–catalyst orientation in ice. The influence of a fast proton transfer in nucleophilic or general base catalysis of β-lactam cleavage is based on the exceptionally high proton mobility known to exist in ice [10]. Enhanced hydroxylaminolysis of simple amide bonds (i.e. acetamide, propionamide, glutamine, asparagine) in ice was reported by the same authors [11]. They suggested that the dielectric behaviour (the dielectric constant of ice being markedly lower than that of water) of ice may facilitate a concerted attack on the substrate by favouring the association of nucleophile molecules.

Butler and Bruice [12] found the spontaneous hydrolysis of acetic anhydride and β-propiolactone to be depressed in ice compared to water. Since water acts as a reactant, its removal in the form of ice could be expected to decrease the reaction rate. The rate enhancements in acid- and base-catalysed hydrolytic reactions were explained by the concentration of the reactants in liquid regions between the ice crystals. Another striking effect was observed in the morpholinolysis of thiolactons in frozen systems where a change in kinetic order as well as an acceleration of the reaction took place [13]. The participation of the ice crystal surface as replacement of a catalyst was also discussed.

The binding of fluoride ions to hemeproteins in frozen systems and the redistribution of solute which occurs during freezing of protein solutions was studied by Yang and Brill [14]. As reported by Taborsky [15], bands of increasing concentration of expelled solute accompany the growth of ice crystals. In a more recent investigation, Takenata *et al.* [16] reported that the oxidation of nitrite by dissolved oxygen to form nitrate which occurs very slowly in solution was accelerated markedly by freezing. The authors excluded ice surface catalysis as an explanation and suggested that separation of ions was responsible for the increase of the reaction rate.

An excellent review of non-enzymatic reactions influenced by freezing of the reaction medium was given by Fennema [17]. The rate enhancements cited there were discussed to be almost completely explainable on the basis of the concentration of the reactants in the unfrozen phase. This 'freeze-concentration effect' was also described by Pincock and Kiovsky [18–21] who investigated reactions in frozen organic solvents and developed equations for expressing reaction rates in the liquid phase of partially frozen solutions.

In aqueous solutions, the 'freeze-concentration effect' is the result of conversion of water into ice crystals of a rather high degree of purity during freezing. When the solution is frozen in a temperature range wherein no eutectics are formed, all non-aqueous constituents are concentrated in a diminished liquid phase which is in equilibrium with the solid solvent. Freeze-concentration can result in supersaturated solutions in which the reactants are concentrated in 0.1% of the original liquid volume [22]. Under these conditions, the molal concentration of the unfrozen phase will depend only on the temperature. At a given temperature, an initially dilute solution will form a large amount of ice whereas a solution of high initial concentration will contain a small amount of ice. Thus even a compound which is not involved in the reaction and has

no effect on the reaction rate of an unfrozen system can have a striking effect on the reaction in frozen state, acting only to increase the liquid phase and thereby diluting the concentration of the reactants.

Enzyme-catalysed Reactions

The influence of freezing on enzyme-catalysed reactions has been summarized by Fennema [23]. When the reaction temperature is lowered and freezing occurs, the reaction rate may follow extrapolation of Arrhenius plots of above freezing data or deviate in both directions. Negative deviations can be explained by formation or stabilization of intramolecular hydrogen bonds in the enzyme, association of the enzyme into polymeric units or increased hydrogen bonding between substrate and water [24]. Contrary to this behaviour, some enzyme-catalysed reactions can be accelerated by freezing, indicating that the Arrhenius effect can be overcompensated by the rate-enhancing effect of a freeze-induced concentration of reactants.

For a bimolecular reaction ($A + B \rightarrow$ products) the reaction rate in the unfrozen liquid phase is given by [21]:

$$dA/dt = -k_2 C[A][B]$$

This equation relates the rate of a frozen solution reaction (measured in the thawed mixture) to the concentration of solutes in the thawed solution. It is pointed out that concentration of the solution by freezing increases the reaction rate by the factor C which is dependent on temperature and the concentration of the reactants before freezing. When the reaction temperature is lowered, C will increase and, especially in solutions with initial low reactant concentrations, a substantial increase in reaction rate will be observed [21].

Enzyme activity in partially frozen systems can be influenced by the nature of the enzyme and the type of freezing treatment. Furthermore, the increase in solute concentration during freezing can result in marked changes of factors such as pH, ion strength and viscosity, which have important influences on enzyme activity and stability. In frozen reaction mixtures, changes in pH can also be caused by crystallization of salts (eutectic formation). Viscosity is increased by ice formation much more then would be expected from the relationship between viscosity and temperature when no phase change occurs [24]. Therefore, diffusion behaviour in frozen systems can be altered. A theoretical analysis of diffusion-controlled reactions in frozen solutions has been reported recently [25].

Grant and Alburn [26] reported that the trypsin-catalysed reactions of hydroxylamine with lysine methyl, lysine ethyl and arginine methyl esters were more rapid in a frozen solution at -23 °C than in a liquid solution at $+1$ °C. They also observed that freezing changed the relative rate of hydrolysis and hydroxylaminolysis in favour of hydroxylaminolysis.

The fact that hydroxylaminolysis of amino acid esters follows the same mechanism as kinetically controlled peptide synthesis initiated studies in our group on the influence of freezing on protease-catalysed peptide synthesis [27].

PROTEASE-CATALYSED PEPTIDE SYNTHESIS IN ICE

Application of Serine Proteases

In 1990 Schuster *et al.* [27] reported that in chymotrypsin-catalysed condensations of Mal-Tyr-OEt with various peptides, amino acid amides and amino acids as nucleophiles, freezing of the aqueous reaction mixtures could significantly increase peptide yield in kinetically controlled synthesis. They demonstrated the use of the method in preparative synthesis and discussed the influence of temperature on the aminolysis/hydrolysis ratio. Peptide yields tended to decrease at temperatures above -10 °C. The yield increasing effect was shown to be especially significant when the amino components used were generally considered to be inefficient nucleophiles in enzyme-catalysed reactions at room temperature. These results were confirmed by other investigators ([28], Table 1).

Schuster *et al.* [29] later discussed these results in relation to the 'freeze-concentration model' and concluded that it should be a reasonable basis for the explanation of the yield-increasing effect in protease-catalysed peptide synthesis.

In contrast to amino acid amides and peptides, unprotected amino acids cannot donate hydrogen bonds supporting a non-covalent acyl enzyme–nucleophile complex, required for an efficient acyl transfer [3]. Surprisingly, under frozen state conditions the endoproteinase chymotrypsin is capable of coupling even free amino acids, acting as a reverse carboxypeptidase [27, 30–32].

Chymotrypsin acylated various amino acids in a reaction using 2 mM Mal-Phe-OMe and 50 mM (50% as free base) of the appropriate amino acid at -25 °C in unexpectedly high yields (% given in brackets): Ala (70), Met (75), Val (58), Ser (52), Ile (35),

Table 1 α-Chymotrypsin-catalysed Synthesis of Model Peptides at 25 °C and in Frozen Aqueous Solutions

Acyl donor	Peptide	Peptide yield (%)		Reference
		25 °C	Ice	
Mal-Tyr-OMe	Mal-Tyr-Ala-Ala-OH	10	94	[27]
	Mal-Tyr-Gly-Ala-OH	6	95	
	Mal-Tyr-Gly-Gly-Gly-OH	5	91	
	Mal-Tyr-D-Leu-NH_2	10	73	
Ac-Tyr-OEt	Ac-Tyr-Leu-NH_2	82	86	[28]
	Ac-Tyr-His-NH_2	62	84	
	Ac-Tyr-Val-NH_2	74	83	
	Ac-Tyr-Gly-Gly-OH	3	64	

Table 2 α-Chymotrypsin-catalysed Peptide Synthesis at 25 °C and in Frozen Aqueous Solutions using N^{α}- unprotected Acyl Donors

Acyl donor	Peptide	Yield (%)		Reference
		25 °C	Ice	
H-Phe-OMe	H-Phe-Leu-NH_2	20	48	[2]
	H-Phe-Arg-NH_2	58	75	
	H-Phe-Arg-OH	0	77	
	H-Phe-Ala-Ala-NH_2	22	64	
H-Tyr-OEt	H-Tyr-Lys-OH	0	71	[32]
	H-Tyr-Gln-OH	0	61	
	H-Tyr-Ser-OH	0	78	
	H-Tyr-Thr-OH	0	50	

Thr (30), Asn (29), Leu (26), Lys (60), Arg (13) and Phe (17) [30, 31]. The authors also reported the synthesis of Mal-Phe-Gly-OH on a gram scale with a yield of 47%, using only two equivalents of glycine. The observed low reactivity of amino acids with bulky hydrophobic side chains was explained by the restricted mobility of bulky structures in the unfrozen liquid phase. Similar results in coupling Mal-Tyr-OEt with free amino acids were obtained by Tougu *et al.* [32]. They also investigated the dependence of peptide yield on pH of the reaction mixture before freezing. Peptide yields increased by rais' ıg pH up to an optimum (pH 10.5) corresponding to the almost complete deprotonization of the α-amino groups of the nucleophiles. Higher pH values resulted in decreased peptide yields likely due to dissociation of an ionizable group of the enzyme. The specificity of the tyrosyl–chymotrypsin aminolysis by free amino acids in frozen solution was found to be substantially different from that by the corresponding amides in solution. Successful coupling of free amino acids as nucleophiles in chymotrypsin-catalysed reactions seems to depend strongly on the conditions used. Whereas the condensation of Ac-Tyr-OEt with Arg and Lys in frozen reaction mixtures gave yields of 40% and 36%, respectively, the coupling of His, Leu, Val and Gly failed [28].

In 1992 we established that the protection of the N^{α}-amino group of the acyl donor ester is not necessary in chymotrypsin-catalysed peptide synthesis in frozen aqueous systems [30]. N^{α}-unprotected acyl donor esters were also used in further studies [2, 32]. Examples are given in Table 2. The results obtained using N^{α}-unprotected amino acid esters as acyl donors indicate an unexpected substrate specificity of chymotrypsin in the sense of a reverse aminopeptidase. The successful coupling of N^{α}-unprotected aromatic amino acid esters with free amino acids opens the possibility of a very simple strategy of peptide synthesis which cannot be performed by chemical methods. A single step synthesis of kyotorphin in frozen solution catalysed

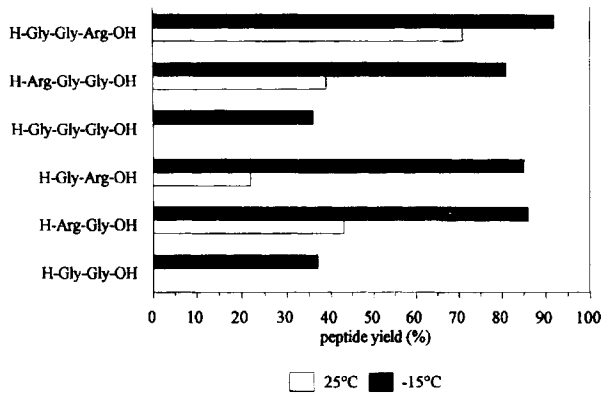

Figure 1 Comparison of the S'-specificity of α- chymotrypsin using H-Leu-Phe-OMe as acyl donor and a series of di- and tripeptides as amino components at 25 °C and − 15 °C: 10 mM H-Leu-Phe-OMe, 120 mM nucleophile, pH 8.1, 0.4 μM chymotrypsin (25 °C), 4 μM chymotrypsin (− 15 °C).

by chymotrypsin was described by Meos *et al.* [33]. In this process no protecting and deprotecting steps were involved and H-Tyr-Arg-OH was obtained in a yield of about 80%.

This strategy could be extended to N^{α}-unprotected dipeptide esters as acyl donors. H-Asp-Phe-Ala-Ile-OH was synthesized under semipreparative conditions in a yield of 86%. Furthermore, the S'-specificity of α-chymotrypsin in frozen aqueous state was investigated using H-Leu-Phe-OMe as acyl donor and a series of di- and tripeptides as nucleophiles (Figure 1) (S. Gerisch and H.-D. Jakubke, submitted for publication). As predicted by subsite mapping [34], the enzyme shows a preference for positively charged amino components in P'_1- and P'_3-position but not in P'_2 for reactions at room temperature (S'-subsite nomenclature according to [35]). In frozen aqueous solution a changed S'-subsite specificity of α-chymotrypsin for peptide bond formation was observed.

N^{α}-unprotected unusual acyl donors were also used in enzyme-catalysed peptide synthesis [36]. Most of the uncoded acyl donors tested were shown to be well-accepted substrates for the endopeptidase α-chymotrypsin. Various N^{α}-unprotected unusual phenylalanine ester derivatives were coupled with H-Leu-NH_2 and H-Arg-NH_2. Compared with reactions at room temperature, a significant yield-increasing effect of freezing could be established (Table 3).

Under normal reaction conditions amino acid alkyl esters show only moderate nucleophilic efficiency because the possibility of hydrogen bond formation with the S'-binding sites of the enzyme is lacking [3]. Furthermore, the lability of ester bonds to enzymatic cleavage can cause undesired side reactions. Schuster *et al.* [29] reported that these drawbacks can be overcome by freezing the reaction mixture. In the synthesis of Mal-Phe-Leu-OMe freezing caused both an increase of peptide yield and a suppression of secondary conversion of the product. Using amino acid alkyl esters as amino components, the resulting peptide can serve as acyl donor in the next protease-catalysed reaction step. The method of freezing opens the possibility for an enzyme-catalysed step-by-step peptide synthesis without the necessity for intermediate manipulations of the carboxyl terminus of the acyl donor.

Besides chymotrypsin, which has been studied most frequently, relatively few data about peptide synthesis catalysed by other serine proteases in frozen systems have been reported [7, 28, 27]. The coupling of Ac-Tyr-OEt with various nucleophiles catalysed by carboxypeptidase Y in ice did not result in yield improvement in the case of amino acid amides, but a consistent decrease of yield for the free amino acids was demonstrated by Littlemore *et al.* [28]. Using Bz-Arg-OEt as acyl donor and trypsin as catalyst, the same authors reported an advantageous effect of freezing for amino acid amides. Trypsin-catalysed coupling of free amino acids as nucleophiles in frozen solution failed with the exception of Arg and Lys which provide yields of 38% and 18% in ice, respectively.

The trypsin-catalysed condensation of Pht-Ala-OBzl with H-Leu-NH_2 with 91% yield in frozen solution compared with 42% at room temperature indicates a change in P_1-specificity of trypsin in frozen systems [7]. Subtilisin (Carlsberg) and alcalase from *Bacillus licheniformis* were successfully used in peptide synthesis in frozen solutions [7]. Endopeptidase Glu C from *Staphylococcus aureus* (V8 protease) was able to catalyse the condensation of Z-Glu-OMe with H-Ala-Ala-OH with 76% yield in ice whereas at room temperature the yield was only 5% [27].

Application of Cysteine Proteases

Owing to their low cost and the ability to form acyl enzyme intermediates, cysteine proteases are suitable catalysts in kinetically controlled peptide synthesis.

Schuster *et al.* [27] obtained a significantly higher yield in the papain-catalysed synthesis of Mal-Phe-Ala-Ala-OH in ice starting from Mal-Phe-Ala-OEt(Cl) and H-Ala-Ala-OH, compared with the reaction at room temperature. As reported by Littlemore *et al.*

Table 3 α-Chymotrypsin-catalysed Dipeptide Amide Synthesis Using Unusual N^{α}-unprotected Donors (from [36])

Substrate	Peptide	Peptide yield (%)	
		25 °C	− 15 °C
H-Phe-OMe	H-Phe-Leu-NH_2	52	94
	H-Phe-Arg-NH_2	79	98
H-4-fluoro-Phe-OMe	H-4-fluoro-Phe-Leu-NH_2	47	90
	H-4-fluoro-Phe-Arg-NH_2	73	95
H-4-nitro-Phe-OMe	H-4-nitro-Phe-Leu-NH_2	86	94
	H-4-nitro-Phe-Arg-NH_2	90	98
H-2-naphtyl-Ala-OMe	H-2-naphtyl-Ala-Leu-NH_2	55	93
β-phenyllactyl-OMe	β-phenyllactyl-Leu-NH_2	78	98
	β-phenyllactyl-Arg-NH_2	92	100

25 mM acyl donor, 100 mM nucleophile, pH 9.0.

[28], papain-catalysed condensations of Bz-Arg-OEt with various amino components in frozen reaction mixtures resulted in both increases and decreases of peptide yield. When using amino acid amides as nucleophiles, yields tended to be smaller at − 20 °C. Lys gave higher yields in the frozen state whereas yields decreased in coupling of Leu and Arg and coupling of His, Val and Gly failed. In contrast, we observed a general yield-increasing effect of freezing in papain-catalysed reactions, probably caused by using different reaction conditions ([5], results discussed below).

By using the synthesis of Bz-Arg-Leu-NH_2 as a model reaction, we showed the capability of ficin from *Ficus carica latex* to form peptide bonds in frozen aqueous solution [37]. The influence of temperature, amino component concentration and pH before freezing was studied. Whereas the dependence of peptide yield on temperature and amino component concentration was similar to that observed in α-chymotrypsin-catalysed reactions in ice [29] the pH dependence of the reaction exhibited a different behaviour. Peptide yields increased up to an optimum at pH 7.8. The increase in unprotonated N^{α}-amino groups at pH values above the optimum did not result in higher peptide yields. Possibly, a change in pH can take place by freezing with effects on enzyme structure. Alterations in pH of aqueous buffer solutions during freezing have been reporrted [38, 39]. A similar dependence of peptide yield on pH of the reaction mixture was obtained in papain- and clostripain-catalysed synthesis of Bz-Arg-Leu-NH_2 in the frozen state [5].

Furthermore, we compared the catalytic effect of ficin, papain and clostripain from *Clostridium histolyticum* on the condensation of Bz-Arg-OEt with various amino acid amides and dipeptides in ice under optimized reaction conditions [5]. Owing to its restricted specificity for Arg-X-bonds including proline in P'_1-position [40, 41], the application of clostripain to peptide synthesis is of particular interest. The results obtained demonstrate the positive influence of freezing the reaction mixture but the increase in peptide yield in the frozen state differs according to the enzyme and the nucleophile used (Table 4). With the exception of H-Pro-NH_2 and H-D-Leu-NH_2, in papain- and ficin-catalysed reactions a general yield increasing effect of freezing was observed indicating a change in S'-specificity of both enzymes by freezing the reaction system. The lack of binding with H-D-Leu-NH_2 could not be overcome in ice. Concerning this behaviour, there is a marked difference between the cysteine proteases ficin and papain and the serine protease chymotrypsin, which showed remarkably higher yields in coupling of nucleophiles with D-configuration in frozen solutions compared with the inefficient coupling at room temperature [27, 42]. Using clostripain, H-Pro-NH_2 and H-D-Leu-NH_2 could be coupled according to the enzyme specificity and in agreement with the results of S'-subsite mapping at room temperature reported by Ullmann and Jakubke [43]. In contrast to the other cysteine and serine proteases studied, clostripain shows no change in specificity in frozen reaction mixtures. This behaviour could be caused by the heterodimeric structure of clostripain [44] which

Table 4 Protease-catalysed Condensation of Bz-Arg-OEt with Amino Acid Amides and Dipeptides (from [5])

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

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

differs from the other proteases used for catalysing peptide bond formation in ice.

INFLUENCE OF MODIFIED REACTION CONDITIONS ON PEPTIDE SYNTHESIS IN FROZEN AQUEOUS SYSTEMS

The chymotrypsin-catalysed reaction of Mal-Phe-OMe with H-Leu-NH_2 has been studied under a range of reaction conditions, for example decreased reaction temperature, addition of organic solvent and various cryogenic reagents used for shock freezing [42]. The reaction temperature was studied down to − 60 °C, based on the observation that a liquid water phase persists in a protein–water system down to about − 70 °C [45]. The optimal reaction temperature was found between − 10 and − 25 °C. At lower temperatures peptide yields decreased, indicating that retardation of reaction rates predominates over the influence of freeze-concentration.

In order to ensure homogeneous solutions before freezing, the substrates have to be completely dissolved in the aqueous medium. The use of solubilizing N^{α}-protecting groups of the acyl donor ester has been described [27, 29–32, 37]. Solubility problems can also be overcome by the addition of water-miscible organic solvents. The synthesis of Mal-Phe-Leu-NH_2 was therefore studied in various mixtures of water and dimethyl sulphoxide at sub-zero temperatures [42]. As expected, the addition of more than 10% of the organic solvent resulted in decreased peptide yields. This effect was explained as the result of a distorted ice structure in which the advantages of the concentration of the reactants have been lost.

Enzymatic peptide synthesis in ice described so far has employed the use of liquid nitrogen for shock freezing which is connected with the 'Leidenfrost phenomenon'. An object inserted in liquid nitrogen is momentarily protected against the cold by a gas layer, possibly allowing the formation of ice structures. To evaluate the influence of this phenomenon, liquid nitrogen, liquid propane and an isopropanol/dry ice system were compared as cryogenic reagents. It was shown that the reaction course is independent of the shock freezing reagent.

WHICH FACTORS ARE RESPONSIBLE FOR THE YIELD-INCREASING EFFECT OF FREEZING IN PROTEASE-CATALYSED PEPTIDE SYNTHESIS?

Gerisch *et al.* [42] established that enzyme-catalysed peptide synthesis in ice is only possible when an active enzyme is used as catalyst. They studied the influence of different enzymes and proteins (bovine serum albumin, lysozyme, insulin, casein and phenylmethylsulphonyl fluoride-treated chymotrypsin) on the reaction of Mal-Phe-OMe with H-Leu-NH_2 in frozen aqueous solutions. It was found that these proteins are not able to catalyse peptide bond

Table 5 Ratio [Mal-Tyr-Leu-NH_2]/[Mal-Tyr-OH] in the Thermodynamic Equilibrium of α-Chymotrypsin-catalysed Reactions (from [46])

T (°C)	State of aggregation	[Mal-Tyr-Leu-NH_2]/[Mal-Tyr- OH]
20	Liquid	0.076 ± 0.001
5.5	Liquid	0.067 ± 0.005
0	Liquid	0.067 ± 0.005
− 4.5	Liquid	0.050 ± 0.006
− 9	Liquid	0.050 ± 0.008
− 4.5	Frozen	0.34 ± 0.03
− 9	Frozen	0.54 ± 0.04

0.5 mM Mal-Tyr-OH, 20 mM H-Leu-NH_2 (effective concentration), 0.18 mM chymotrypsin.

formation by unspecific surface catalysis. In the presence of bovine serum albumin, Mal-Phe-OMe was hydrolysed but no peptide synthesis occured. Using chymotrypsin specifically inhibited by phenylmethylsulphonyl fluoride, no peptide bond formation was observed. Similar observations were made by Littlemore *et al.* [28].

To explain the yield-increasing effect of freezing, Schuster *et al.* [29] considered the 'freeze-concentration model' a reasonable basis. They also demonstrated the influence of freezing of aqueous reaction mixtures on the equilibrium of α-chymotrypsin-catalysed thermodynamically controlled peptide synthesis ([46], Table 5). Since the equilibrium is independent of enzyme properties, the results can be attributed to the effect of freezing the reaction mixture. The experiments performed in supercooled solutions demonstrate that the significant effect of freezing on the equilibrium ratio cannot be a result of the decreased reaction temperature. The shift of equilibrium in favour of the peptide formation in frozen solutions can be explained by the 'freeze-concentration' of the reactants in the unfrozen liquid phase.

The question arises whether concentration of the reactants is the only reason for yield enhancement by freezing. As already outlined above, involvement of other physical and reaction parameters, like imposition of a favourable orientation of substrate and catalyst [9], increased proton mobility in ice [10], catalysis by ice surface [13], changes of dielectric behaviour [9] and reduced water activity [31] have been discussed. Physical and chemical alterations of protein structure and function, caused by freezing aqueous protein solutions, may also play a role in this field. An overview was given by Taborsky [47], who emphasized the effects of low temperature and freezing on hydrophobic interactions, hydrogen binding and interactions of the protein with the solvent, other solution components and the ice–liquid interface. Low temperatures were expected to alter protein conformations by favouring the formation of and strengthening hydrogen bonds and diminishing the importance of hydrophobic interactions. The freezing-induced concentration of protein ligands may result in significant conformation changes even with ligands that would bind too weakly to have an effect under dilute conditions. The author also discussed the influence of freezing on protein hydration.

In a recent report, Tougu *et al.* [48] studied the dependence of peptide yield on concentration of reactants and other added components in order to establish the involvement of concentration effects in peptide yield enhancement by freezing. The hyperbolic dependence of peptide yield (*Y*) on nucleophile concentration ([N]) which these authors obtained in all cases was discussed according to a kinetic equation based on the reaction scheme for kinetically controlled peptide synthesis:

$$Y = Y_{max}[N]/K_{app} + [N]$$

As expected from the fact that K_{app} involves a parameter of nucleophile binding while Y_{max} does not, freezing resulted in a marked decrease of K_{app} while Y_{max} remained unaffected. Added substances which are not involved in the reaction, such as NaCl or methanol, decreased peptide yields. The authors suggested that this decrease is the result of nucleophile dilution due to an increase in the volume of the liquid microinclusions of ice. Peptide yields in frozen systems and in highly concentrated solutions were compared to prove the dominant role of freeze-concentration in peptide yield enhancement (Table 6). From these data shown the authors concluded that concentrated solutions match the reaction

Table 6 Peptide Yields in Chymotrypsin-catalysed Reaction of Mal-Tyr-OEt with Free Amino Acids (from [48])

Amino	Concentration	Peptide yield (%)		
acid	(%)	+20 °C (liquid)[a]	− 18 °C (liquid)[a]	− 18 °C (frozen)[b]
Glu	40	10	18	18
Gly	55	-	85	85
Lys	47	58	86	84
Ala	30	75	85	84
Thr	37	82	87	92

[a] 20 mM Mal-Tyr-OEt.
[b] 2 mM Mal-Tyr-OEt, nucleophile concentration 1/10 of that in liquid.

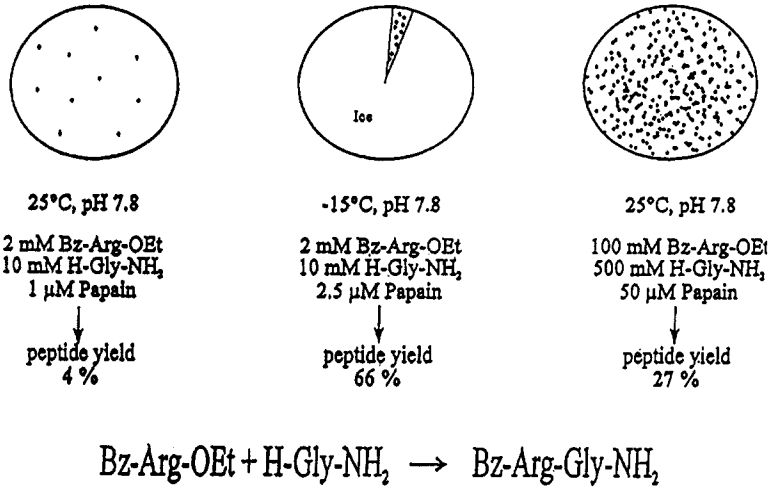

Figure 2 Papain-catalysed synthesis of Bz-Arg-Gly-NH_2 starting from different substrate concentrations at room temperature and in frozen state (from [7]).

conditions in the unfrozen liquid phase in ice and no changes occur with the enzyme.

However, specific properties of peptide synthesis reactions in frozen systems, such as changes in specificity of the protease which were observed in a number of studies [5, 31, 32] suggest that there probably exist other factors involved in peptide yield enhancement by freezing. This assumption is supported by recent investigations reported by Jakubke *et al.* [7]. In order to estimate the freeze-concentration factor, the ^{1}H-NMR relaxation time technique has been used to determine the amount of unfrozen water in frozen reaction samples at − 15 °C. An apparent concentration factor of 50 for the unfrozen water concentration was obtained by comparison with relaxation time measurements for the same system at room temperature. To simulate these concentration relations, papain-catalysed peptide synthesis experiments were carried out in high concentration solutions at room temperature (Figure 2). From the results obtained the authors concluded that freeze- concentration cannot be the only cause of the yield-increasing effect because high concentration reactant solutions cannot simulate the reaction conditions in frozen systems.

CONCLUDING REMARKS

Frozen state protease catalysis opens up completely new possibilities for peptide synthesis, including coupling of nucleophiles considered ineffective in

solution, the application of a minimum protection strategy and suppression of competitive reactions in the reversal of proteolysis. The synthetic potential of serine and cysteine protease-catalysed peptide synthesis has therefore been considerably increased. Currently, the possibility of a further scaling-up beyond gram scale is under investigation. In the growing market for peptide pharmaceuticals enzyme-catalysed peptide synthesis in frozen aqueous systems could contribute to the improvement of the synthetic tools and meet economical as well as ecological requirements.

The specific properties of enzyme-catalysed reaction systems in frozen aqueous solutions justify further investigation of the role of factors other than the concentration effect in yield enhancement by freezing. The unexpected catalytic behaviour in protease-catalysed reactions in the frozen state might also be valid for reactions catalysed by other enzymes. Unpublished results indicate that freezing the aqueous reaction mixture can also increase the yield in synthetic reactions catalysed by other hydrolases like β-galactosidase and ribonuclease T_1.

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