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Reverse action of hydrolases in frozen aqueous solutions

Review Article

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Summary. The reverse action of hydrolases provides an attractive alternative to the chemical synthesis of peptides, oligosaccharides and oligonucleotides. Freezing the reaction mixture has proved to suppress competitive reactions in enzyme-catalysed peptide synthesis. After a short discussion of the influence of freezing on enzyme-catalysed reactions the current manuscript gives an overview of protease-catalysed peptide synthesis and the possible reasons of the yield-enhancing effect of freezing. The application of glycosidases and ribonucleases for synthetic purposes in frozen reaction mixtures is summarized.

Keywords: Amino acids – Synthesis – Frozen Aqueous Solution – Protease – Glycosidase – Ribonuclease

Abbrevations: Ac, acetyl; Bz, benzoyl; C>p, cytidine 2′:3′-cyclic monophosphate; CpU, cytidylyl (3′-5′)uridine; DTT, dithioerythritol; Mal, maleyl; NH_2, amide; OBzl, benzyl ester; OEt, ethyl ester; OEt(Cl), monochloroethyl ester; OMe, methyl ester; pNA, p-nitroanilide; Pht, phthalyl; Z, benzyloxycarbonyl; U, uridine

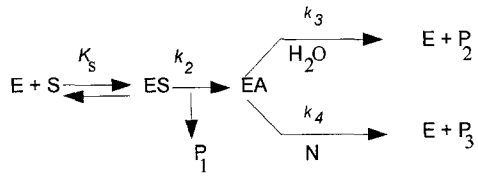
Introduction

The growing demand for peptides, oligosaccharides, and oligonucleotides of biological and pharmaceutical interest requires the development of efficient synthetic methods. Although classical chemical methods in solution, solid-phase synthesis, and recombinant techniques play an important role in peptide synthesis, all these methods suffer from several drawbacks. While the main problems of chemical synthesis are racemization and the necessity for a side chain protecting/deprotecting strategy, the incorporation of unnatural amino acids is limited when recombinant DNA techniques are used. The chemical synthesis of carbohydrates as polyfunctional, chiral substances

requires a well-designed protective group strategy and tailored activation approaches. These methods, as well as the chemical synthesis of RNA fragments, are quite laborious and time-consuming. The application of enzymes for synthetic purposes should provide an attractive alternative. Generally, enzymes operate in a substrate-, stereo- and regiospecific manner without the need of protective group chemistry. Suitable tools for biomolecule synthesis should be enzymes which catalyse their formation *in vivo*. The major problem is the complexity of many of these enzyme systems which require coordination with numerous factors. Isolation, purification, and stabilization of those enzymes are often extremely difficult and expensive. The only alternative to native ligases are hydrolases due to the principle of microscopic reversibility. Synthetic applications of proteases (Jakubke, 1987, 1994; Bongers and Heimer, 1994), glycosidases (Nilsson, 1988; Ichikawa et al., 1992; Monsan and Paul, 1995; Halcomb, 1995), and ribonucleases (Mohr and Thach, 1969; Bauer et al., 1972; Rowe and Smith, 1972; Bratovanova et al., 1993; Backmann et al., 1994) have been reported. In enzymatic peptide synthesis two strategies have been developed (for reviews, see Jakubke, 1987, 1994; Schellenberger and Jakubke, 1991; Bongers and Heimer, 1994). The equilibrium-controlled approach corresponds to the direct reversal of hydrolysis with the equilibrium shifted to the thermodynamically more stable cleavage products. In the kinetic approach, a transient accumulation of the peptide product far above the equilibrium concentration can be achieved during the transfer of acyl donor groups to nucleophilic acceptor molecules in serine and cysteine protease-catalysed peptide synthesis (Scheme 1).

Glycosidase-catalysed formation of glycosydic bonds is quite analogous to protease-catalysed peptide synthesis. Glycosidases are able to catalyse either the direct coupling of glycosyl moieties by simple reversion of the hydrolysis reaction or the transfer of a glycosyl residue from an activated donor onto an acceptor molecule (Nilsson, 1988; Ichikawa et al., 1992; Monsan and Paul, 1995; Halcomb, 1995).

In dinucleoside monophosphate synthesis catalysed by several ribonucleases only the application of the kinetically controlled approach has been reported (Mohr and Thach, 1969; Bauer et al., 1972; Rowe and Smith, 1972; Bratovanova et al., 1993; Backmann et al., 1994). It was shown that ribonucleases can catalyse the synthesis of dinucleoside phosphates from nucleoside 2′:3′-cyclic phosphates and a free nucleoside based on the reversibility of the transesterification step in the breakdown of RNA.



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 Haensler et al., 1995b)

The most important yield limiting factor in kinetically controlled hydrolase-catalysed reactions is the competitive hydrolysis of the acyl enzyme and the noncovalent enzyme-donor intermediate, respectively. In protease-catalysed peptide synthesis, undesired side reactions can be diminished by medium engineering, manipulation of the reaction conditions (Jakubke, 1994), and the use of highly specific acyl donor leaving groups (Schellenberger et al., 1991; Jakubke et al., 1996). Chemically modified enzymes, enzyme mutants, or zymogens as catalysts also play a role in this field (Jakubke, 1994). In glycosidase-catalysed synthetic reactions, the final yield will be increased by using an activated donor which is cleaved more rapidly than the product (Nilsson, 1988). The suppression of competitive hydrolytic reactions by reduction of water concentration in the reaction mixture, by addition of organic solvents, or the use of micro-aqueous monophasic organic solvents has been demonstrated in peptide synthesis (Jakubke, 1994). In kinetically controlled formation of glycosydic bonds this effect has not generally been observed (Halcomb, 1995). Furthermore, freezing can also reduce the water concentration of the reaction mixture. A decrease of the proteolysis rate caused by freezing was reported by Lineweaver (1939). Therefore, freezing should result in a suppression of competitive hydrolytic reactions in enzyme-catalysed synthesis of peptides, oligosaccharides, and oligonucleotides.

The behaviour of enzyme-catalysed reactions in frozen reaction mixtures

The influence of freezing on enzyme-catalysed reactions has been summarized by Fennema (1975a). Below the freezing point of the reaction mixture, the reaction rate may follow extrapolation of Arrhenius plots of above freezing data or deviate in both directions. 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 (Dozou, 1971) can cause negative deviations. In contrast, freezing can also result in a substantial acceleration of the reaction. Freeze-induced rate enhancements have also been observed in nonenzymatic reactions (Fennema, 1975b). Pincock and Kiovsky (1965a–c, 1966), who investigated reactions in frozen organic solvents, attributed the rate-enhancing effect of freezing to the concentration of the reactants in the liquid phase of partially frozen solutions. In aqueous solutions, freezing induces the growth of ice crystals of a rather high degree of purity. In a temperature range wherein no eutectics are formed, all solutes are concentrated in a diminished liquid phase which can amount to 0.1% of the original liquid volume (Franks, 1985) and is in equilibrium with the solid solvent. At a given temperature, the molar concentration of the reactants in the liquid phase is determined by the concentration of the solutes before freezing. All non-aqueous components of the solution, even components which are not involved in the reaction, influence the volume of the liquid phase. Therefore, freeze-concentration can result in overcompensation of the Arrhenius effect, especially in solutions with initially

low reactant concentrations wherein a large amount of ice will be formed during freezing. Diffusion behaviour in frozen systems can be altered because viscosity is increased by ice formation much more then would be expected from the relationship between viscosity and temperature when no phase change occurs (Dozou, 1971). A theoretical analysis of diffusion-controlled reactions in frozen solutions has been reported recently (Parker and Ring, 1995). Factors which mainly influence enzyme activity such as pH, ion strength, and viscosity can be changed by the increased solute concentration during freezing. Crystallization of salts (eutectic formation) can also cause marked changes of the pH in frozen reaction solutions The type of freezing treatment and the nature of the enzyme can also affect enzyme activity in partially frozen reaction mixtures. The exceptionally high proton mobility known to exist in ice (Eigen and de Mayer, 1958), a favourable substrate-catalyst orientation, the dielectric behaviour of ice (the dielectric constant of ice being markedly lower than that of water) (Grant et al., 1961; Alburn and Grant, 1965), the participation of the ice crystal surface as replacement of a catalyst (Bruice and Butler, 1964), and separation of ions in frozen aqueous systems (Takenata et al., 1992) have been discussed as further factors which influence reactions in ice.

Enzymatic formation of peptide bonds in frozen aqueous systems

Grant and Alburn (1966) observed that in trypsin-catalysed hydroxylaminolysis of amino acid esters freezing changed the relative rate of hydrolysis and hydroxylaminolysis in favour of hydroxylaminolysis. Because these reactions follow the same mechanism as kinetically controlled peptide synthesis studies on the influence of freezing on protease-catalysed peptide synthesis

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-Arg-OH	<2	33	Schuster et al., (1990)
	Mal-Tyr-Lys-OH	<2	44	
	Mal-Tyr-D-Leu-NH_2	10	73	
	Mal-Tyr-Ala-Ala-OH	10	94	
	Mal-Tyr-Gly-Ala-OH	6	95	
	Mal-Tyr-Gly-Gly-Gly-OH	5	91	
Ac-Tyr-OEt	Ac-Tyr-Arg-OH	0	40	Littlemore et al., (1993)
	Ac-Tyr-Lys-OH	10	36	
	Ac-Tyr-His-NH_2	62	84	
	Ac-Tyr-Leu-NH_2	82	86	
	Ac-Tyr-Val-NH_2	74	83	
	Ac-Tyr-Gly-Gly-OH	10	36	

were initiated by Schuster et al. (1990). The authors reported that amino acid amides, peptides, and even free amino acids which are considered to be inefficient nucleophiles at room temperature provide significantly higher yields at −25°C (Table 1). These results were confirmed by Littlemore et al. (1993) (Table 1). Schuster et al. (1991) established that the "freeze-concentration model" should be also a reasonable basis for the explanation of the yield-increasing effect of freezing observed in protease-catalysed peptide synthesis.

It is surprising that under frozen state conditions the endoproteinase chymotrypsin is capable of coupling even free amino acids acting as a reverse carboxypeptidase (Schuster et al., 1990, 1993a; Ullmann and Jakubke, 1993; Tougu et al., 1993). Various amino acids were acylated by chymotrypsin starting from 2mM Mal-Phe-OMe and 50mM (50% as free base) of the appropriate amino acid at −25°C in unexpectedly high yields (% given in brackets): Met (75), Val (58), Ser (52), Ile (35), Thr (30), Asn (29), Leu (26), Lys (60), Arg (13), and Phe (17) (Ullmann and Jakubke, 1993). The synthesis of Mal-Phe-Gly-OH on a gram scale yielded 47%, using only two equivalents of glycine. Amino acids with bulky hydrophobic side chains showed low reactivity, possibly due to their restricted mobility in the unfrozen liquid phase. Tougu et al. (1993) reported similiar results coupling Mal-Tyr-OEt with free amino acids. They found the specificity of the tyrosyl-chymotrypsin aminolysis by free amino acids in frozen reaction mixtures to be substantially different to that by the corresponding amides in solution. However, the reaction conditions of the chymotrypsin-catalysed coupling of free amino acids as nucleophiles seem to be of cruical importance. Littlemore et al. (1993) only succeded in coupling of Ac-Tyr-OEt with Arg and Lys catalysed by chymotrypsin in frozen solutions. His, Leu, Val, and Gly as amino components gave no peptide product.

In 1992 we reported on the successful chymotrypsin-catalysed coupling of N^{α}-unprotected acyl donor esters in frozen aqueous systems (Ullmann and Jakubke, 1993). The unexpected substrate specificity of chymotrypsin in the sense of a reverse aminopeptidase has been underlined by further studies including the use of free amino acids as nucleophiles (Tougu et al., 1993; Jakubke, 1994) (Table 2). This strategy represents the simplest type of peptide synthesis which cannot be performed by chemical methods. Starting from H-Tyr-OEt and Arg, Meos et al. (1993) synthesized kyotorphin in a yield of about 80% in a single step procedure in frozen solution employing chymotrypsin. Furthermore, also N^{α}-unprotected dipeptide esters H-Xaa-Phe-OMe (Jakubke et al., 1996) and even N^{α}-unprotected unusual acyl donors (Gerisch et al., 1995) could be successfully coupled in frozen reaction mixtures (Table 2). Compared to the reactions at room temperature, a significant increase in peptide yield in the frozen state was observed. H-Asp-Phe-Ala-Ile-OH was synthesized in a yield of 86% under semipreparative conditions (Jakubke et al., 1996). The S′-specificity of α-chymotrypsin in frozen aqueous solutions was investigated using H-Leu-Phe-OMe as acyl donor and a series of di- and tripeptides as nucleophiles. The enzyme shows a preference for positively charged amino components in P_1'- and P_3'-position but not in P_2'

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-Tyr-OEt	H-Tyr-Lys-OH	0	71	Tougu et al.,
	H-Tyr-Gln-OH	0	61	(1993)
	H-Tyr-Ser-OH	0	78	
H-Phe-OMe	H-Phe-Arg-OH	0	77	Jakubke, (1994)
	H-Phe-Arg-NH_2	58	75	
	H-Phe-Leu-NH_2	20	77	
H-Gly-Phe-OMe	H-Gly-Phe-Ala-Ile-OH	23	85	Jakubke et al.,
H-Leu-Phe-OMe	H-Leu-Phe-Ala-Ile-OH	5	88	(1996)
H-Asp-Phe-OMe	H-Asp-Phe-Ala-Ile-OH	23	91	
H-4-fluoro-Phe-OMe	H-4-fluoro-Phe-Leu-NH_2	47	90	Gerisch et al.,
H-2-naphtyl-Ala-OMe	H-2-naphtyl-Ala-Leu-NH_2	55	93	(1995)
β-phenyllactyl-OMe	β-phenyllactyl-Leu-NH_2	78	98	

(S′-subsite nomenclature according to Schechter and Berger, 1967) at room temperature (Schellenberger et al., 1994). In contrast, in the frozen state an amino component with arginine at P_2' was as well accepted as nucleophiles with arginine at P_1'- and P_3'-position indicating a changed S′-subsite specificity of α-chymotrypsin for peptide bond formation in ice (Jakubke et al., 1996).

The use of amino acid alkyl esters as nucleophiles in peptide synthesis under normal reaction conditions suffers from several drawbacks. The lacking possibility of hydrogen bond formation with the S′-binding sites of the enzyme results in only moderate nucleophilic efficiency (Schellenberger and Jakubke, 1991). The sensitivity of ester bonds to enzymatic cleavage can cause undesired side reactions. As reported by Schuster et al. (1991), freezing the reaction mixture resulted in both an increase in peptide yield and a suppression of secondary conversion of the product in chymotrypsin-catalysed synthesis of Mal-Phe-Leu-OMe. Using amino acid alkyl esters as amino components, freezing opens the possibility for an enzyme-catalysed step by step peptide synthesis without the necessity for additional manipulation of the carboxyl terminus of the acyl donor because the resulting peptide can serve as acyl donor in the next protease-catalysed reaction step.

Only few data about peptide synthesis catalysed by serine proteases other than chymotrypsin in frozen systems have been reported (Schuster et al., 1991; Littlemore et al., 1993; Jakubke et al., 1996). Littlemore et al. (1993) described an advantageous effect of freezing in trypsin-catalysed coupling of Bz-Arg-OEt with amino acid amides. Using the same catalyst, coupling of free amino acids as nucleophiles in frozen solution failed with the exception of Arg and Lys which provided yields of 38% and 18%, respectively. Using Ac-Tyr-OEt as acyl donor in carboxypeptidase Y-catalysed peptide synthesis,

freezing did not result in yield improvement in the case of amino acid amides, but a consistent decrease in yield for the free amino acids as nucleophiles was demonstrated (Littlemore et al., 1993).

Jakubke et al. (1996) reported the successful use of subtilisin (Carlsberg) and alcalase from *Bac. licheniformis* in peptide synthesis in frozen solutions. Endopeptidase Glu C from *Staphylococcus aureus* (V8 protease) catalyses the condensation of Z-Glu-OMe and H-Ala-Ala-OH with 76% yield in ice whereas at room temperature the yield was only 5% (Schuster et al., 1990).

The application of dipeptidylpeptidase IV (E.C. 3.4.14.5) in peptide synthesis has been reported by Heiduschka et al. (1989). The enzyme separates Xaa-Pro- and Xaa-Ala-dipeptide units from the unblocked N-terminus of a peptide chain. Owing to its substrate specificity, the enzyme represents an interesting catalyst for peptide bond formation. Therefore, we studied the capability of dipeptidylpeptidase IV to catalyse peptide synthesis in frozen aqueous solutions using the condensation of H-Phe-Pro-pNA and glycine as a model reaction. Unfortunately, no peptide bond formation could be observed under these conditions (Haensler and Jakubke, unpublished results). The enzyme exhibited an extremely low hydrolytic activity in the frozen state, possibly due to its size and dimeric structure (total molecular weight about 300,000; Wolf et al., 1978). This fact could indicate a restricted flexibility in the unfrozen liquid phase.

Besides the widely used serine proteases cysteine proteases are suitable catalysts in kinetically controlled peptide synthesis due to their capability to form acyl enzyme intermediates and their low costs. The first application of a cysteine protease in frozen reaction mixtures has been reported by Schuster et al. (1990) who used papain to synthesize a model peptide. In papain-catalysed condensations of Bz-Arg-OEt with various amino components in frozen reaction mixtures both increases and decreases in peptide yield were observed by Littlemore et al. (1993). While Lys gave higher yields in the frozen state yields tended to be smaller using amino acid amides as nucleophiles at −20°C. Using the same acyl donor, we observed a general yield-increasing effect of freezing in papain-catalysed reactions, probably caused by different reaction conditions (Haensler et al., 1995b, see below).

Ficin from *Ficus carica latex* has also been successfully used to form peptide bonds in frozen aqueous solutions (Haensler et al., 1995a). The influence of pH of the reaction mixture before freezing has been studied in ficin- (Haensler et al., 1995a), papain- and clostripain-catalysed (Haensler et al., 1995b) peptide synthesis. In cysteine protease-catalysed reactions, the pH dependence of peptide yield exhibited a behaviour different from that observed in chymotrypsin-catalysed peptide bond formation in ice (Schuster et al., 1991). Peptide yields increased to an optimum at pH 7.8 (ficin, papain) and 7.0 (clostripain), respectively. The increase of unprotonated N^{α}-amino groups at pH values above the optimum did not result in higher peptide yields, possibly caused by a change of pH during freezing as reported by William-Smith et al. (1977) and Orii and Morita (1977) with effects on enzyme structure.

Clostripain from *Clostridium histolyticum* exhibits a restricted specificity for Arg-X-bonds including proline in P_1'-position (Ogle and Tytell, 1953; Mitchell, 1968). For this reason, clostripain represents a very interesting catalyst in peptide synthesis. We studied the influence of freezing on clostripain-catalysed condensation of Bz-Arg-OEt with various amino acid amides and dipeptides compared to ficin and papain catalysis under optimized reaction conditions. The results obtained clearly demonstrate that the increase in peptide yield in the frozen state depends on the enzyme and the nucleophile used (Table 3). In papain- and ficin-catalysed reactions a general yield increasing effect of freezing (with the exception of H-Pro-NH_2 and H-D-Leu-NH_2 which could not be coupled at all) was observed indicating a change in S'-specificity of both enzymes. There is an important difference between the cysteine proteases ficin and papain and the serine protease chymotrypsin concerning the lack of affinity to H-D-Leu-NH_2. For the latter protease remarkably higher yields were obtained coupling nucleophiles with D-configuration in frozen solutions compared to the inefficient coupling at room temperature (Schuster et al., 1990; Gerisch et al., 1994). Using clostripain, H-Pro-NH_2 and H-D-Leu-NH_2 could be coupled according to the results of S'-subsite mapping at room temperature reported by Ullmann and Jakubke (1994). The results indicate, in contrast to the other cysteine and serine proteases studied, an unchanged specificity of clostripain in frozen reaction mixtures. This behaviour is possibly attributed to the heterodimeric structure of clostripain (Gilles et al., 1984) which differs from the other proteases.

The influence of reaction conditions like temperature, organic solvent, and shock freezing on peptide synthesis in frozen aqueous solutions has been investigated by Gerisch et al. (1994). They found the optimal reaction temperature in chymotrypsin-catalysed reactions between −10°C and −25°C. At lower temperatures, decreased peptide yields were observed due to a

Table 3. Protease-catalysed condensation of Bz-Arg-OEt with amino acid amides and dipeptides (from: Haensler et al., 1995b)

Nucleophile	Peptide yield (%)					
	Ficin		Papain		Clostripain	
	25°C	−15°C	25°C	−15°C	25°C	−15°C
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-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-Pro-OH	12	35	9	78	40	42

[Bz-Arg-OEt] = 2 mM, [nucleophile] = 10 mM (free base), pH 7.8 (papain, ficin), pH 7.0 (clostripain).

retardation of the reaction that predominates over the influence of freeze-concentration. Lower peptide yields obtained in reaction mixtures containing more than 10% of organic solvent (dimethyl sulfoxide) were explained as a result of a distorted ice structure which has a striking effect on the concentration of the reactants. The authors also tested various shock freezing reagents (liquid nitrogen, liquid propane and an isopropanol/dry ice system) and found that the course of the reaction was independent on the cryogenic reagent used.

Only few reports concerning the role of freeze-concentration in yield-enhancement of enzyme-catalysed peptide synthesis have been published up to now. Schuster et al. (1991), who explained the yield increasing effect of freezing on the basis of the "freeze-concentration model", also reported a shift of equilibrium in α-chymotrypsin-catalysed thermodynamically controlled peptide synthesis (Schuster et al., 1993b). In addition, they studied peptide synthesis in supercooled solutions and demonstrated that the significant effect of freezing on the equilibrium ratio cannot be alone a result of the decreased reaction temperature.

Recently, Tougu et al. (1995) discussed the involvement of freeze-concentration in peptide yield enhancement on the basis of kinetic equations. They found that nucleophile binding was markedly influenced by freezing. Furthermore, peptide yields in highly concentrated solutions and in frozen reaction mixtures were compared to prove the dominant role of freeze-concentration in peptide yield enhancement. Almost equal yields were obtained in frozen systems and in 10-fold concentrated solutions at room temperature. The authors concluded that the reaction conditions in the liquid micro-inclusions of a frozen aqueous system correspond to that in concentrated solutions and no changes occur with the enzyme.

However, the changes in specificty of the proteases observed in frozen systems (Schuster et al., 1993a; Tougu et al., 1993; Haensler et al., 1995b) cannot be explained by freeze-concentration of the reactants only. Obviously, there exist other factors which contribute to this effect. Using the ^{1}H-NMR relaxation time technique, the amount of unfrozen water has been determined in frozen samples at -15°C and compared to relaxation time measurements for the same system at room temperature (Jakubke et al., 1996). An apparent concentration factor of 50 for the unfrozen water concentration was obtained. Papain-catalysed peptide synthesis experiments were carried out in highly concentrated solutions at room temperature in order to simulate these con-

Table 4. Papain-catalysed synthesis of Bz-Arg-Gly-NH_2 starting from different substrate concentrations at room temperature and in frozen state (from Jakubke et al., 1996)

T (°C)	[Papain] (μM)	[Bz-Arg-OEt] (mM)	[H-Gly-NH_2] (mM)	Peptide yield (%)
25	1	2	10	4
−15	2.5	2	10	66
25	50	100	500	25

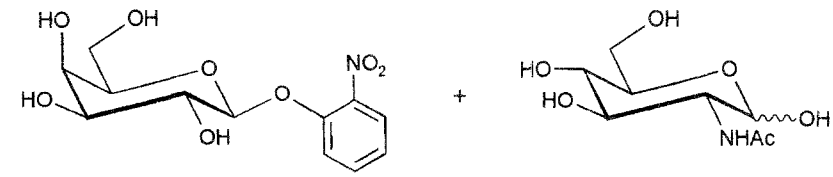
centration relations (Table 4). The results obtained indicate that highly concentrated reactant solutions cannot simulate the reaction conditions in frozen systems. The authors concluded that freeze-concentration cannot be the only cause of the yield-increasing effect.

A summary of physical and chemical alterations of protein structure and function, caused by freezing aqueous protein solutions which may also be of importance in this field was given by Taborsky (1979). The author discussed the influence of freezing on protein hydration, hydrogen bonding, interactions of the protein with the solvent, other solution components, and the ice-liquid interface. Protein conformation is influenced by low temperatures favouring the formation and strengthening of hydrogen bonds and diminishing the importance of hydrophobic interactions. Significant conformational changes can be caused by freezing-induced concentration of protein ligands. As already discussed above, other physical and reaction parameters like imposition of a favourable orientation of substrate and catalyst (Grant et al., 1961), increased proton mobility in ice (Eigen and de Mayer, 1958), catalysis by ice surface (Bruice and Butler, 1964), changes of dielectric behaviour (Alburn and Grant, 1965), and reduced water activity (Schuster et al., 1993a) may also be involved in the yield-enhancing effect of freezing.

Reverse action of glycosidases and ribonuclease A in frozen aqueous solutions

There are close similiarities between kinetically controlled syntheses of glycosides and peptides which both involve condensation of an activated donor and a nucleophilic acceptor. On the other hand, the hydroxyl group is less nucleophilic than the amino group. Therefore, glycoside synthesis will be less favoured than peptide bond formation. Yields in kinetically controlled glycoside synthesis generally range from 20 to 40% (Nilsson, 1988; Ichikawa et al., 1992; Halcomb, 1995). Besides the relatively low yields, the main drawback of glycosidase-catalysed synthesis is the low regioselectivity of these enzymes. Regioselectivity can vary with different enzymes and careful selection of the catalyst can afford the formation of the desired linkage (Ajisaka et al., 1994). β-Galactosidase from *E. coli* (E.C. 3.2.1.23) formed $\beta 1 \rightarrow 6$-linkages exclusively. Reports concerning the use of this enzyme in glycoside synthesis have been summarized in several reviews (Nilsson, 1988; Ichikawa et al., 1992; Halcomb, 1995).

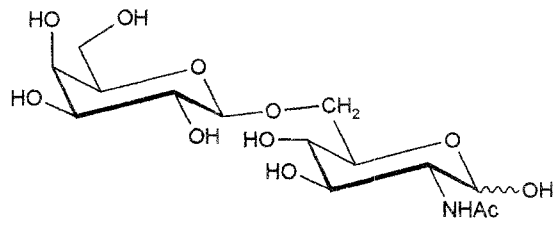
In order to investigate the effect of freezing on β-galactosidase-catalysed synthetic reactions, we studied the synthesis of β-D-galactopyranosyl-N-acetyl-D-glucosamine at 25°C and at −5°C (Scheme 2) (Haensler and Jakubke, unpublished results). The samples were stirred at 25°C or incubated in a kryostate at −5°C after shock freezing in liquid nitrogen. The reaction course was monitored by HPLC using an amino-phase column and UV detection at 235 nm. Variations of the pH of the reaction mixture before freezing did not improve the yields. Although the yields obtained in frozen state were higher than in solution, the yield-enhancing effect of freezing was less distinct as in protease-catalysed peptide synthesis. This is possibly due to the rela-



o-nitrophenyl-β -D-galactopyranoside
(50 mM)

+

N-acetyl-D-glucosamine
(100 mM)



β -D-galactopyranosyl-N-acetyl-D-glucosamine
yield: 35 % (25°C) ; 53 % (-5 °C)

Scheme 2. β-Galactosidase-catalysed synthesis of β-D-galactopyranosyl-N-acetyl-D-glucosamine from o-nitrophenyl-β-D-galactopyranoside and N-acetyl-D-glucosamine. 0.1 M potassium phosphate buffer pH 6.5 (1 mM $MgCl_2$, 5 mM DTT); enzyme concentration: 0.6 U/ml (25°C), 30 U/ml (−5°C); reaction time: 3 h (25°C), 8 h (−5°C)

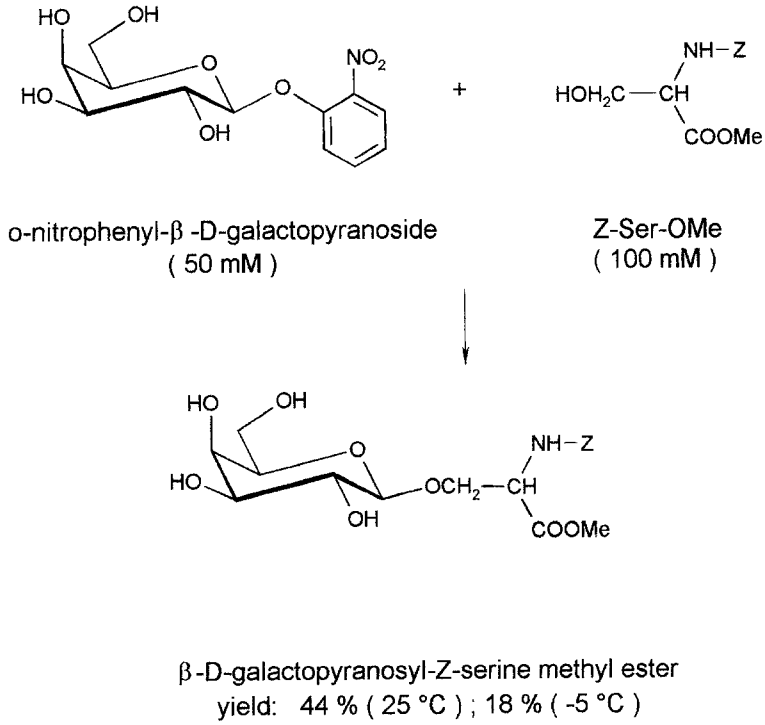
tively high reaction temperature (−5°C) that influences the degree of freeze-concentration. However, β-galactosidase exhibited only very low activity at temperatures below −5°C. This behaviour can probably be caused by a restricted flexibility of the tetrameric enzyme with a total molecular weight of about 465,000 (Huber et al., 1994) in the liquid microinclusions of a frozen reaction mixture.

In further experiments, we studied the capability of α-glucosidase from brewers yeast (E.C. 3.2.1.20) to catalyse the synthesis of maltose from p-nitrophenyl-α-D-glucopyranoside and D-glucose in solution as well as in frozen state. The reaction mixtures containing 25 mM p-nitrophenyl-α-D-glucopyranoside, 50 mM glucose in phosphate buffer pH 7.5, and different enzyme concentrations (given in brackets) were incubated at 25°C (0.6 U/ml), 0°C (22.5 U/ml), and after shock freezing at −5°C (75 U/ml). The donor hydrolysis was monitored spectrophotometrically at 420 nm. The concentration of glucose was determined by the glucoseoxidase/peroxidase method (Hugget and Nixon, 1957). The disaccharide yield was calculated from the decrease in glucose concentration. At all reaction temperatures studied a disaccharide yield of about 20% was obtained (Haensler and Jakubke, unpublished results). At −5°C the reaction occured extremely slow but the danger of donor hydrolysis during freezing prevented a further increase in enzyme concentration. After 20 hours the reaction mixture contained still about 60%

of the donor. Considering this result, the disaccharide yield of 22% indicates a yield-increasing effect of freezing which is, however, concealed by the low reaction rate.

Furthermore, glycosidases are also capable to transfer glycosyl moieties of an activated donor to side chain hydroxyl groups of amino acid derivatives and peptides (Cantacuzene and Attal, 1991; Sauerbrei and Thiem, 1992; Holla et al., 1992; Attal et al., 1992; Nilsson and Scigelova, 1994; Leparoux et al., 1994). β-D-Galactopyranosyl-L-serine represents the linkage region of many O-glycosylated biologically active glycoproteins and thus its synthesis is of great interest. The synthesis of several β-D-galactopyranosyl-serine derivatives catalysed by β-galactosidase from *E.coli* in yields ranging from 10 to 40% has been reported by Cantacuzene and Attal (1991) and Holla et al. (1992).

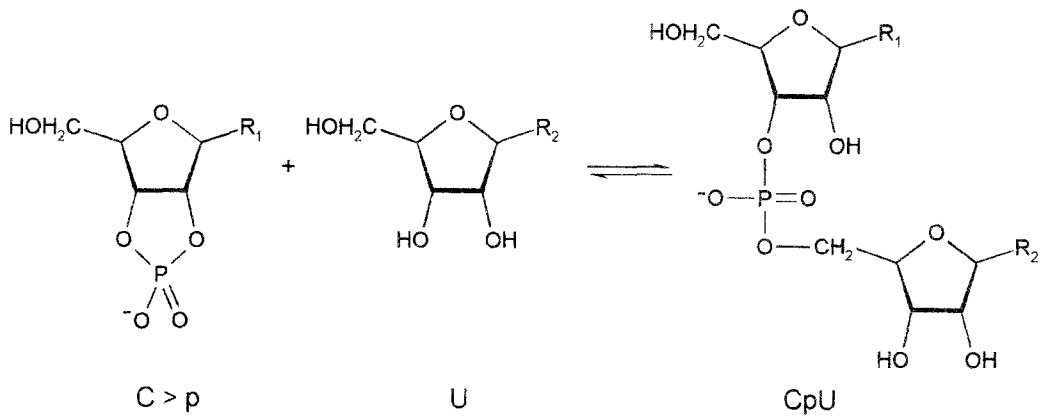
We studied the influence of freezing on the formation of linkages between a sugar und the hydroxyl group of an amino acid derivative using the synthesis of β-D-galactopyranosyl-Z-serine methylester catalysed by β-galactosidase from *E.coli* as a model reaction (Scheme 3) (Haensler and Jakubke, unpublished results). The reactions were monitored by HPLC using a reversed phase RP 18 column and UV detection at 254 nm. A lower yield was obtained in the frozen state compared to the reaction in solution at 25°C. The variation of the pH before freezing (from 6.0 to 8.0) and of the acceptor concentration did not improve the yield. Due to its lower cleavage rate, lactose gave lower



Scheme 3. β-Galactosidase-catalysed synthesis of β-D-galactopyranosyl-Z-serine methylester from o-nitrophenyl-β-D-galactopyranoside and Z-Ser-OMe. 0.1 M potassium phosphate buffer pH 7.5 (1 mM $MgCl_2$, 5 mM DTT); enzyme concentration: 1 U/ml (25°C), 50 U/ml (−5°C); reaction time: 6 h (25°C), 24 h (−5°C)

yields as galactosyl donor both in frozen state and in solution. Similiar results were obtained using α-glucosidase from brewers yeast as catalyst and p-nitrophenyl-α-D-glucopyranoside as donor. Obviously, the yield-enhancing effect of freezing in glucosidase-catalysed syntheses seems to depend strongly on the nucleophilic acceptor used.

Ribonuclease A catalyses the breakdown of RNA in a two step process involving transphosphorylation of the 3′- 5′-phosphodiester bond resulting in the formation of a 2′:3′-cyclic phosphodiester and its hydrolysis to a 3′-monophosphate group (for a review, see Nogues et al., 1995). Due to the reversibility of the transphosphorylation step, ribonuclease A can catalyse the synthesis of dinucleoside monophosphates from 2′:3′-cyclic phosphates and free nucleosides (Mohr and Thach, 1969; Bratovanova et al., 1993). In this reaction, alcoholysis of the 2′:3′-cyclic phosphate by the nucleoside and hydrolysis by water are competing reactions. Mohr and Thach (1969) found a synthesis/hydrolysis ratio in ribonuclease A-catalysed synthesis substantially smaller than 1. In the hope of reducing the hydrolysis rate, we studied the synthesis of cytidylyl(3′-5′)uridine catalysed by ribonuclease A from bovine pancreas in frozen aqueous solutions (Scheme 4). The synthetic reactions were carried out starting from different concentrations of the reactants after shock freezing at −10°C and 0°C. A strong dependence of dinucleoside phosphate synthesis on enzyme concentration has been reported in ribonuclease T_1-catalysed reactions (Rowe and Smith, 1970). Therefore, we studied the reaction at varying enzyme concentrations. The results are shown in Table 5 (Haensler and Jakubke, unpublished results). In conclusion, in reaction mixtures with an initially low reactant concentration improved dinucleoside monophosphate yields could be obtained. Starting from higher substrate concentrations, freezing resulted in lower yields. In initially dilute reaction mixtures a larger amount of ice will be formed, according to the freeze-concentration model, compared to more concentrated solutions in which the reactants will be less concentrated. The dinucleoside phosphate yields obtained proved to be independent on the enzyme concentration.



Scheme 4. Ribonuclease A-catalysed synthesis of cytidylyl-(3′-5′)uridine from cytidine 2′:3′-cyclic monophosphate and uridine. *C > p*, cytidine 2′:3′-cyclic monophosphate, *U*, uridine, *CpU*, cytidylyl(3′-5′)uridine; R_1, cytosine, R_2, uracile

Table 5. Ribonuclease A-catalysed synthesis of cytidylyl-(3′-5′)uridine in solution and in frozen state

[RNAse A] (μg/ml)	[C > p] (M)	[U] (M)	CpU yield (%)	
			0°C	−10°C
100	0.00275	0.35	18	30
200			20	32
350			17	30
200	0.25	1	28	n.d.
500			28	19
700			29	22
2,000			n.d.	20

C > p: 2′:3′-cyclic cytidine monophosphate, U: uridine, CpU: cytidylyl(3′-5′)uridine 0.1 M Tris/HCl, pH 7.0, reaction time: 24 h (0°C), 72–96 h (−10°C).

Concluding remarks

Freezing the reaction mixture can significantly increase the yields in hydrolase-catalysed synthetic reactions. The possibility of coupling nucleophiles which are inefficient in solution and the application of a minimum protection strategy as well as the suppression of side reactions substantially increase the synthetical potential of serine and cysteine protease catalysed peptide synthesis.

While most of the proteases studied proved to be excellent catalysts in frozen aqueous systems, mixed results were obtained using other hydrolases. Size and structure of the enzyme seem to influence its catalytic behaviour in frozen reaction mixtures. The effect of freeze-concentration can be overlayed by large reductions in reaction rate. These results clearly indicate that the yield-increasing effect of freezing is not a predictable general phenomenon in the reversal of enzyme-catalysed hydrolytic reactions but strongly depends on the nature of the catalyst and the reactants. Further investigations are required to clarify the role of factors other than the concentration effect in yield enhancement by freezing.

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References

Alburn HE, Grant NH (1965) Reactions in frozen systems. II. Enhanced hydroxylaminolysis of simple amides. J Am Chem Soc 87: 4174–4177

Ajisaka K, Fujimoto H, Isomura M (1994) Regioselective transglycosylation in the synthesis of oligosaccharides: comparison of β-galactosidases and sialidases of various origins. Carbohydr Res 259: 103–115

Attal S, Bay S, Cantacuzene D (1992) Enzymatic synthesis of β-galactosyldipeptides and of β-1,3-digalactosylserine derivatives using β-galactosidase. Tetrahedron 48: 9251–9260

Backmann J, Doray CC, Grunert HP, Landt O, Hahn U (1994) Extended kinetic analysis of ribonuclease T_1 variants leads to an improved scheme for the reaction mechanism. Biochem Biophys Res Commun 199: 213–219

Bauer S, Lamed R, Lapidot Y (1972) Large scale synthesis of dinucleoside monophosphates catalysed by ribonuclease from *Aspergillus clavatus*. Biotechnol Bioengineer 14: 861–870

Bongers J, Heimer EP (1994) Recent applications of enzymatic peptide synthesis. Peptides 15: 183–193

Bratovanova EK, Kasche V, Petkov DD (1993) Kinetically controlled enzymic synthesis of ribonucleotide bond in vitro. Biotechnol Lett 15: 347–352

Bruice TC, Butler AR (1964) Catalysis in water and ice. II. The reaction of thiolactons with morpholine in frozen systems. J Am Chem Soc 86: 4104–4108

Cantacuzene D, Attal S (1991) Enzymic synthesis of galactopyranosyl-L-serine derivatives using galactosidases. Carbohydr Res 211: 327–331

Dozou P (1971) Aqueous-organic solutions of enzymes at subzero temperatures. Biochimie 53: 1135–1145

Eigen M, de Mayer L (1958) Self-dissociation and protonic charge transport in water and ice. Proc Roy Soc (London) A247: 505–533

Fennema O (1975a) Activity of enzymes in partially frozen aqueous systems. In: Duckworth RB (ed) Water relations of food. Proceedings of the International Workshop, Glasgow 1974. Academic Press, London, pp 397–412

Fennema O (1975b) Reaction kinetics in partially frozen aqueous systems. ibid., pp 539–556

Franks F (1985) Biophysics and biochemistry at low temperatures. Cambridge University Press, Cambridge, p 61

Gerisch S, Ullmann G, Stubenrauch K, Jakubke HD (1994) Enzymatic peptide synthesis in frozen aqueous systems: Influence of modified reaction conditions on the peptide yield. Biol Chem Hoppe-Seyler 375: 825–828

Gerisch S, Jakubke HD, Kreuzfeld HJ (1995) Enzymatic peptide synthesis in frozen aqueous systems: use of N^{α}-unprotected unusual acyldonors. Tetrahedron: Assymetry 6: 3039–3049

Gilles AM, Lecroisey A, Keil B (1984) The primary structure of α-clostripain light chain. Eur J Biochem 145: 469–476

Grant NH, Alburn HE (1966) Acceleration of enzyme reactions in ice. Nature 212: 194

Grant NH, Clark DE, Alburn HE (1961) Imidazole- and base-catalysed hydrolysis of penicillin in frozen systems. J Am Chem Soc 83: 4476–4477

Haensler M, Keilhack H, Jakubke HD (1995a) Die Verwendung von Ficin zur Knüpfung von Peptidbindungen im gefrorenen wäßrigen System. Pharmazie 50: 184–187

Haensler M, Ullmann G, Jakubke HD (1995b) The application of papain, ficin and clostripain in kinetically controlled peptide synthesis in frozen aqueous solutions. J Peptide Sci 1: 283–287

Halcomb RL (1995) Hydrolysis and formation of C-O-bonds. Glycosidases. In: Drauz K, Waldmann H (eds) Enzyme catalysis in organic sythesis. VCH Verlagsgesellschaft mbH, Weinheim, pp 303–306

Heiduschka P, Dittrich J, Neubert K, Barth A (1989) Die Verwendung der Dipeptidylpeptidase IV zur Knüpfung der Peptidbindung. Pharmazie 44: 778–780

Holla EW, Schudok, M, Weber A, Zulauf, M (1992) Enzyme-catalysed synthesis of O-glycosylated peptide building blocks. J Carbohydr Chem 11: 659–663

Huber RE, Gupta MN, Khare SK (1994) The active site and mechanism of the β-galactosidase from *E.coli*. Int J Biochem 26: 309–318

Hugget ASD, Nixon DA (1957) Enzymatic detection of blood glucose. Biochem J 66: 12P

Ichikawa Y, Look GC, Wong CH (1992) Enzyme-catalysed oligosaccharide synthesis. Anal Biochem 202: 215–238

Jakubke HD (1987) Enzymatic peptide synthesis. In: Udenfried S, Meienhofer J (eds) The peptides: analysis, synthesis, biology. Academic Press, New York, pp 103–165

Jakubke HD (1994) Protease-catalysed peptide synthesis: basic principles, new synthesis strategies and medium engineering. J Chin Chem Soc 41: 355–370

Jakubke HD, Eichhorn U, Gerisch S, Haensler M, Salchert K, Ullmann G, Ullmann D (1996) Nonconventional approaches to enzyme-catalysed peptide synthesis. In: Wilcox CS, Hamilton AD (eds) Molecular design and bioorganic catalysis. Proceedings of the NATO Advanced Workshop on Bioorganic Catalysis, Johnstown, USA, 1995. Kluwer Academic Publishers, pp 53–70

Leparoux S, Fortun Y, Colas B (1994) Synthesis of β-galactosyl-(hydroxy amino acid) derivatives using β-galactosidase activity from *Achatina achatina* digestive juice. Biotechnol Lett 16: 677–682

Lineweaver H (1939) The energy of activation of enzymatic reactions and their velocity below 0°C. J Am Chem Soc 61: 403–407

Littlemore L, Schober P, Widmer F (1993) Proteases, ice and peptide bonds. In: Yanaihara N (ed) Peptide chemistry. Proceedings 2nd Japan Symposium on Peptide Chemistry, Escom, Leiden, pp 185–187

Meos H, Tougu V, Haga M, Aaviksaar A, Schuster M, Jakubke HD (1993) Single step synthesis of kyotorphin in frozen solutions by chymotrypsin. Tetrahedron: Assymetry 4: 1559–1564

Mitchell WM (1968) Hydrolysis at arginyl-proline in polypeptides by clostridiopeptidase B. Science 162: 374–375

Mohr SC, Thach RE (1969) Application of ribonuclease T_1 to the synthesis of oligoribonucleotides of defined base sequence. J Biol Chem 244: 6566–6576

Monsan P, Paul F (1995) Enzymatic synthesis of oligosaccharides. FEMS Microbiol Rev 16: 187–192

Nilsson KGI (1988) Enzymatic synthesis of oligosaccharides. TIBTECH 6: 256–264

Nilsson KGI, Scigelova M (1994) Synthesis of glycosylated serine and threonine derivatives employing glycosidases. Biotechnol Lett 16: 671–676

Nogues MV, Vilanova M, Cuchillo CM (1995) Bovine pancreatic ribonuclease A as a model of an enzyme with multiple substrate binding sites. Biochim Biophys Acta 1253: 16–24

Ogle JD, Tytell AA (1953) The activity of *Clostridium histolyticum* proteinase on synthetic substrates. Arch Biochem Biophys 42: 327–336

Orii Y, Morita M (1977) Measurement of the pH of frozen buffer solutions by using pH indicators. J Biochem 81: 163–168

Parker R, Ring SG (1995) A theoretical analysis of diffusion controlled reactions in frozen solutions. Cryo-Lett 16: 197–208

Pincock RE, Kiovsky TE (1965a) Bimolecular reactions in frozen organic solutions. J Am Chem Soc 87: 2072–2073

Pincock RE, Kiovsky TE (1965b) Reactions in frozen solutions. II. Base-catalysed decomposition of *t*-butylperoxyformate in frozen *p*-xylene. J Am Chem Soc 87: 4100–4107

Pincock RE, Kiovsky TE (1965c) Reactions in frozen solutions. III. Methyl iodide with triethylamine in frozen benzene solutions. J Am Chem Soc 88: 51–55

Pincock, RE, Kiovsky TE (1966) Kinetics of reactions in frozen solutions. J Chem Educ 43: 358–360

Rowe MJ, Smith MA (1970) Synthesis of 3′–5′-dinucleotides with RNAse T_1. Biochem Biophys Res Commun 38: 393–399

Rowe MJ, Smith MA (1972) Synthesis of oligoribonucleotides with ribonuclease T_1. Biochim Biophys Acta 281: 338–346

Sauerbrei B, Thiem J (1992) Galactosylation and glucosylation by use of β-galactosidase. Tetrahedron Lett 33: 201–204

Schechter I, Berger A (1967) On the size of the active site of proteinases. I. Papain. Biochem Biophys Res Commun 27: 157–162

Schellenberger V, Jakubke HD (1991) Protease-catalysed kinetically controlled peptide synthesis. Angew Chem, Int Ed Engl 30: 1437–1449

Schellenberger V, Goerner A, Koennecke A, Jakubke HD (1991) Protease-catalysed peptide synthesis. Prevention of side reactions in kinetically controlled reactions. Pept Res 4: 265–269

Schellenberger V, Turck CW, Rutter WJ (1994) Role of the S'subsites in serine protease catalysis. Active site mapping of rat chymotrypsin, rat trypsin, α-lytic protease and a cercarial protease from *Schistosoma mansoni.* Biochemistry 33: 4251–4257

Schuster M, Aaviksaar A, Jakubke HD (1990) Enzyme-catalysed peptide synthesis in ice. Tetrahedron 46: 8093–8102

Schuster M, Aaviksaar A, Haga M, Ullmann U, Jakubke HD (1991) Protease-catalysed peptide synthesis in frozen aqueous systems: The "freeze-concentration model". Biomed Biochim Acta 50: 84–89

Schuster M, Ullmann G, Jakubke HD (1993a) Chymotrypsin-catalysed peptide synthesis in ice: use of unprotected amino acids as acyl acceptors. Tetrahedron Lett 34: 5701–5702

Schuster M, Jakubke HD, Aaviksaar A (1993b) Thermodynamically controlled α-chymotrypsin-catalysed peptide synthesis in frozen aqueous solutions. In: Brandenberg P, Ivanow VT, Voelter W (eds) Chemistry of peptides and proteins, vols 5/6, DWI Reports, vols 112 A/B, pp 203–209

Taborsky G (1979) Protein alterations at low temperatures: an overview. In: Fennema O (ed) Proteins at low temperatures. Advances in chemistry series. Am Chem Soc, Washington, No 180, pp 1–26

Takenaka N, Ueda A, Maeda Y (1992) Acceleration of the rate of nitrite oxidation by freezing in aqueous solution. Nature 358: 736–738

Tougu V, Meos H, Haga M, Aaviksaar A, Jakubke HD (1993) Peptide synthesis by chymotrypsin in frozen solutions. Free amino acids as nucleophiles. FEBS Lett 329: 40–42

Tougu V, Talts P, Meos H, Haga M, Aaviksaar A (1995) Aminolysis of acyl-chymotrypsin by amino acids. Kinetic appearance of concentration effect in peptide yield enhancement by freezing. Biochim Biophys Acta 1247: 272–276

Ullmann D, Jakubke HD (1994) The specificty of clostripain from *Clostridium histolyticum.* Mapping the S'subsites via acyl transfer to amino acid amides and peptides. Eur J Biochem 223: 865–872

Ullmann G, Jakubke HD (1993) Frozen aqueous systems: new efficient reaction media for enzyme-catalysed peptide bond formation. In: Schneider CH, Eberle AN (eds) Peptides 1992. Proceedings of the 22th European Peptide Symposium, Interlaken 1992. Escom, Leiden, pp 36–37

William-Smith DL, Bray RC, Barber MJ, Tsopanakis AD, Vincent SP (1977) Changes in apparent pH on freezing aqueous buffer solutions and their relevance to biochemical electron-paramagnetic-resonance spectroscopy. Biochem J 167: 593–600

Wolf B, Fischer G, Barth A (1978) Kinetische Untersuchungen an der Dipeptidylpeptidase IV. Acta Biol Med Germ 37: 409–420

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