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Temperature effects on protease catalyzed acyl transfer reactions in organic media

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Abstract

The influence of reaction temperature on synthesis activity, product yield and nucleophile specificity for α-chymotrypsin and subtilisin Carlsberg were studied. The enzymes were immobilized on Celite and used in acetonitrile with a water content of 10%. Acyl-transfer reactions with Ac–PheOEt as acyl donor and 11 different amino acid amides and 3 dipeptides as nucleophiles were studied. The decrease in temperature from 25 to −1°C had a positive effect on the peptide yield in all reactions studied. The most efficient nucleophiles for the two enzymes α-chymotrypsin and subtilisin Carlsberg is arginine amide and glycine amide, respectively. When decreasing the reaction temperature the yield for α-chymotrypsin increased from 86 to 94% with arginine amide as nucleophile and for subtilisin the yield increased from 75 to 84% for glycine amide.

The nucleophile specificity was determined as the p value, which describes the competition between nucleophile and water for the acyl enzyme. α-Chymotrypsin showed preference for both small and positively charged amino acid residues and subtilisin preferred small uncharged nucleophiles. The temperature did not affect the specificity order but all nucleophiles became more effective in the competition with water at low temperature. In addition, the results indicate that the temperature effect is more pronounced for the smaller nucleophiles.

Keywords: Cryoenzymology; Low temperature; Subtilisin Carlsberg; α-Chymotrypsin; Organic solvent; Peptide synthesis

1. Introduction

The use of proteases as catalysts for peptide synthesis in organic media is an area of active research [1–3]. One advantage of using organic solvent compared to water in protease catalyzed peptide synthesis is that undesired hydrolytic side reactions can be suppressed, and consequently, higher peptide yields are obtained. Another way to increase the peptide yield is to decrease the reaction temperature. In the literature, there are a few reports of high peptide yields in organic media at low temperature [4–7]. The freezing point of the medium is lowered due to the addition of the organic solvent and thus temperatures considerably below 0°C can be used without ice formation. The addition of organic solvent has been used to investigate enzyme kinetics and enzyme mechanisms at subzero temperatures. The low reaction rates at these low temperatures have made it possible to study reaction mechanisms and intermediates [8,9]. The effects of the addition of organic

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solvent on the enzyme mechanism have also been studied at these low temperatures [8,9]. No changes in the enzyme conformation or in the nature of the rate-determining step were found in the temperature range from −45 to 0°C.

The specificity and reactivity of different nucleophiles have been studied in many reports [10–13]. One observed effect when changing from water to organic solvent is that the nucleophile specificity is altered [14–16]. The reactivity of α-chymotrypsin in water is best correlated with the hydrophobicity of the amino acids, and in organic solvents with the normalized Van der Waals volume [16].

The present study focuses on the influence of reaction temperature on nucleophile specificity of two proteases; α-chymotrypsin and subtilisin. Acyl transfer reactions in acetonitrile with 10% of water using Ac–PheOEt as acyl donor have been studied. As nucleophiles amino acid amides and dipeptides with free carboxyl group were used. The synthesis activity and peptide yield for all nucleophiles have been determined.

2. Materials and methods

2.1. Chemicals

α-Chymotrypsin from bovine pancrease (Type II with a specific activity of 50 benzoyl-tyrosine ethyl ester U/mg solid), subtilisin Carlsberg from *Bacillus licheniformis* (Type VIII with a specific activity of 12.3 tyrosine U/mg solid), triethylamine (TEA) and *N*-acetyl-L-phenylalanine ethyl ester (Ac–PheOEt) were obtained from Sigma Chemicals (St. Louis, MO, USA).

Celite (30–80 mesh) was purchased from BDH (Poole, UK) and acetonitrile was of HPLC grade and came from Labscan (Dublin, Ireland).

All amino acid amides and dipeptides were of L-configuration, unless indicated otherwise, and they were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). The amino acid amides were all used as the hydrochlorides, except for the leucine amide which was in the form of free base.

The other chemicals used were of analytical grade.

2.2. Preparation of support

Firstly, Celite was washed with ethanol and purified water. The support was then mixed with 5% HNO_3 and stirred at 80°C for 4 h. The acid-washed Celite was then extensively washed with purified water until pH of the water became neutral. Finally, the Celite was dried at 80°C overnight.

2.3. Immobilization of enzyme

The enzymes were immobilized by being dried onto the support. α-chymotrypsin or subtilisin was dissolved in 50 mM Tris–HCl buffer, pH 7.8 (15 mg/ml), and mixed with Celite (1 ml solution/g). The preparation was then dried under vacuum overnight [17]. All of the enzyme dissolved in the buffer was deposited on the support.

2.4. Enzymatic peptide synthesis

2.4.1. Activity and product yield measurements

Immobilized enzyme and substrate solution (2 ml) were incubated separately at the actual reaction temperature overnight. The reaction was started by adding 50 mg of immobilized enzyme to the reaction medium, which contained acetonitrile with a controlled amount of 50 mM Tris–HCl buffer of pH 7.8, substrates and triethylamine. The initial substrate concentrations were 10 mM Ac–PheOEt and 15 mM nucleophile and the amount of triethylamine was 1% by volume (144 mM) in the final mixture. The reactions were carried out in 4 ml stoppered glass bottles and placed on a head-over-head incubator. The reactions at low temperature were carried out in a cryostat (Arctest) and at room temperature they were placed in a thermostated room (25°C). Samples of 20 μl were taken at

intervals and diluted with 180 μl of the HPLC eluent.

2.4.2. Determination of p value

The constant p describes the competition between nucleophile and water for the acyl–enzyme [18]. The constant corresponds to the nucleophile concentration at which the aminolysis and the hydrolysis rates are the same. The p value was determined by fitting the progress of the reaction according to the following equation:

$$[\mathrm{RCOOH}] = p \ln \frac{[\mathrm{H_2NR'}]_0}{[\mathrm{H_2NR'}]_0 - [\mathrm{RCO{-}NHR'}]} \quad (1)$$

where [RCOOH] is the concentration of hydrolysis product, $[\mathrm{H_2NR'}]_0$ the initial concentration of nucleophile and [RCO–NHR'] the concentration of peptide product.

2.5. Substrate and product analysis

Samples were analyzed by HPLC (Shimadzu SCL-6A) using a C18 column (Spherisorb ODS-2, 10 μm [25 × 0.4 cm], Tracer Analitica), isocratically eluted with water/ acetonitrile/ acetic acid or water/acetonitrile/trifluoroacetic acid and detected spectrophotometrically at 254 nm. The composition when eluting Ac–Phe–Lys-NH_2, Ac–Phe–Arg-NH_2 and Ac–Phe–Ala–Arg-OH was 73/27/0.1 (trifluoroacetic acid) and when eluting Ac–Phe–Ala–Ser-OH the composition was 72/23/5 (acetic acid). All other peptides were eluted with 70/25/5 (acetic acid).

The yields were calculated from peak areas of ester substrate, hydrolysis product, and peptide, which had equal molar extinction coefficients.

3. Results and discussion

It has been shown that higher yields can be obtained in protease catalyzed peptide synthesis when operating at low temperatures [4–7]. One reason for higher yields is that in the competition between the nucleophile and water for the acyl enzyme, the nucleophile is favored by a low temperature. This has been shown for acyl transfer reactions between Ac–PheOEt and Ala-NH_2 catalyzed by α-chymotrypsin [7]. The purpose of this work was to study the influence of

Table 1
Specific activity and peptide yield in α-chymotrypsin catalyzed aminolysis of Ac–PheOEt in acetonitrile containing 10% of water. Reaction conditions as in Fig. 1

Nucleophile	Van der Waals volume [a] (Å^3)	Hydrophobicity [b]	Synthesis activity (μmol/min mg)		Yield [c] (%)	
			25°C	−1°C	25°C	−1°C
Gly-NH_2	48	0	0.230	0.126	72	86
Ala-NH_2	67	−0.39	0.347	0.126	77	88
Ser-NH_2	73	1.24	0.248	0.141	79	91
Asn-NH_2	96	1.91	0.208	0.133	57	74
Thr-NH_2	93	1.00	0.225	0.139	72	87
Val-NH_2	105	−1.30	0.224	0.122	68	84
Leu-NH_2 [d]	124	−1.82	0.253	0.106	57	74
Ile-NH_2	124	−1.82	0.215	0.120	62	80
Lys-NH_2	135	2.77	0.320	0.200	84	94
Arg-NH_2	148	3.95	0.289	0.176	86	94
D-Ala-NH_2	67	−0.39	0.094	0.062	33	44

[a] Volume of individual amino acid residues, enclosed by Van der Waals radius [23].
[b] Calculated from the hydrophobicities of the individual groups that make up each side chain [24].
[c] Peptide yield, when all ester substrate is consumed.
[d] In the form of free base.

reaction temperature on acyl-transfer reactions with different nucleophiles for two proteases; α-chymotrypsin and subtilisin Carlsberg.

3.1. Synthesis activity and peptide yield

The initial synthetic activity and the peptide yield, i.e. the yield obtained when all ester substrate is consumed (this usually corresponds to the point of maximal peptide yield), were determined at two different temperatures, 25 and −1°C. In Table 1 the results for α-chymotrypsin are presented. All synthesis activities at 25°C were high for the nucleophiles tested, meaning that the substrate specificity of chymotrypsin is rather broad. The activity of D-alanine was lower than for the other nucleophiles, but still in the range of acceptable activity. In these reactions the nucleophile competes with water for the deacylation of the acyl–enzyme complex, and two possible products are formed; the peptide and the acid. When changing the reaction temperature from 25 to −1°C the total activity, the sum of the hydrolytic and the synthetic activity for α-chymotrypsin decreased for all nucleophiles by a factor of about 2.5 (data not shown). In the reactions studied, the ratio between synthetic and hydrolytic activity was different for the various nucleophiles, and therefore the decrease in temperature caused varying decreases in synthetic activity (factors between 1.5 and 2.8). The synthesis activities determined at −1°C are all in a range that is acceptable from a practical point of view.

All the peptide yields obtained for the different nucleophiles were high. The general behaviour for all nucleophiles tested was that a decrease in temperature increased the peptide yield. For the most reactive nucleophile the yield increased from 86 to 94% and for the least reactive nucleophile the increase was from 33 to 44% when decreasing the temperature from 25 to −1°C.

The same type of study was made using subtilisin and the results are presented in Table 2. The influence of the reaction temperature on the initial activity was similar to that of chymotrypsin catalyzed reactions. The synthesis activity decreased with a factor between 1.2 and 2.3 for the different nucleophiles. The peptide yield increased with decreasing temperature for all nucleophiles, except for isoleucine (see Table 2). The yields were extremely low for both leucine and isoleucine. The increase in yield for the best nucleophile, glycine, was from 75 to

Table 2
Specific activity and peptide yield for subtilisin catalyzed aminolysis of Ac–PheOEt in acetonitrile containing 10% of water. Reaction conditions as in Fig. 1

Nucleophile	Van der Waals volume [a] (Å^3)	Hydrophobicity [b]	Synthesis activity (μmol/min mg)		Yield [c] (%)	
			25°C	−1°C	25°C	−1°C
Gly-NH_2	48	0	0.233	0.101	75	84
Ala-NH_2	67	−0.39	0.115	0.052	42	46
Ser-NH_2	73	1.24	0.145	0.076	48	60
Asn-NH_2	96	1.91	0.034	0.017	12	14
Thr-NH_2	93	1.00	0.046	0.026	16	20
Val-NH_2	105	−1.30	0.035	0.019	13	14
Leu-NH_2 [d]	124	−1.82	0.007	0.005	3	4
Ile-NH_2	124	−1.82	0.007	0.004	3	3
Arg-NH_2	148	3.95	0.164	0.142	32	33
D-Ala-NH_2	67	−0.39	0.052	0.037	17	26

[a] Volume of individual amino acid residues, enclosed by van der Waals radius [23].
[b] Calculated from the hydrophobicities of the individual groups that make up each side chain [24].
[c] Peptide yield, when all ester substrate is consumed.
[d] In the form of free base.

84% in the temperature range 25 to −1°C. The same amino acid amides as for α-chymotrypsin were used for subtilisin, excepting lysine. It has been reported that the ϵ-amino group of lysine can participate in protease-catalyzed reactions [19]. Indeed, with subtilisin as catalyst both the α-amino group and the ϵ-amino group of lysine amide reacted. Two peptide peaks were obtained in the HPLC chromatograms and for this reason the activity with lysine amide as nucleophile was not determined. α-Chymotrypsin shows preference for the α-amino group, and only one peak of peptide product was obtained, which was assumed to be the α-isomer.

The temperature might influence the pH of the buffer and a change in the apparent pH could cause a change in the amount of uncharged nucleophiles. This could influence the peptide synthesis rate and the peptide yield and therefore we studied the effect of pH at 25 and −1°C (Fig. 1). The synthesis activity at 25°C showed an optimum around pH 8 and at −1°C the activity was unaffected by the pH. The hydrolysis activities were also more or less unaffected by the pH at the two temperatures. It is thus clear that the positive effects of low temperature are not caused by a shift in the pH–activity profile.

The use of a water-poor medium and a low

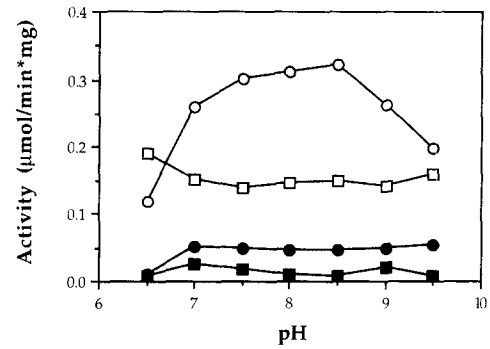

Fig. 1. Effect of pH on initial activity for α-chymotrypsin. The reaction mixture contained 10 mM Ac–PheOEt, 15 mM nucleophile, 1% of triethylamine, 50 mg immobilized α-chymotrypsin preparation and acetonitrile with 10% of buffer. The enzyme was immobilized with 50 mM Tris–HCl buffer at different pH and the same buffer pH was used in the reaction media. Synthesis (open symbols) and hydrolysis (filled symbols) activity were studied at 25°C (circles) and −1°C (squares).

reaction temperature is thus a promising method in enzymatic peptide synthesis. This method is applicable to both α-chymotrypsin and subtilisin with a wide range of amino acid amides as nucleophiles. By decreasing the temperature further the peptide yield was even higher and of course the total activity was decreased. For subtilisin the yield for glycine amide increased to 87% and for α-chymotrypsin to 97% for arginine amide, when decreasing the temperature to −10°C.

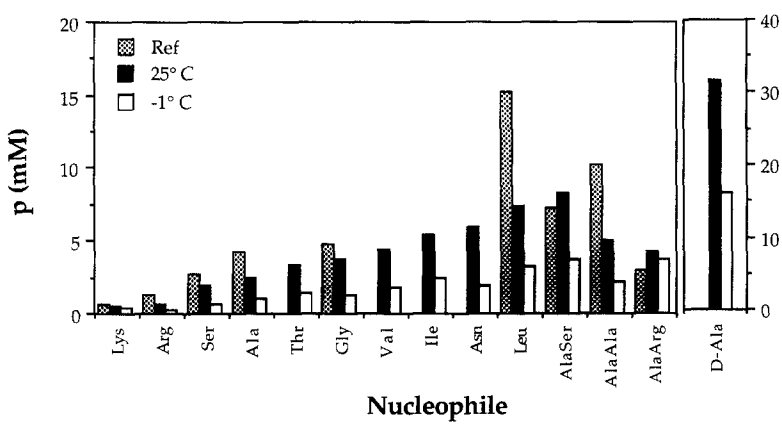

Fig. 2. Nucleophile specificity, p values, for α-chymotrypsin at different temperatures. Reaction conditions were as described in Fig. 1. The reaction was studied at 25 and −1°C and for comparison the values for aminolysis with free α-chymotrypsin using Ac–TyrOEt as acyl donor from Cerovsky and Jakubke (1994) [13] were added.

3.2. Nucleophile specificity

The nucleophile specificity was quantified by measuring the p value, which is defined as the nucleophile concentration at which the aminolysis and the hydrolysis rates are the same [11,18,20]. This value is a measure of the efficiency of the nucleophile compared to water. The aminolysis of Ac–PheOEt with different amino acid amides and dipeptides as nucleophiles was studied in acetonitrile with a content of 10% water at 25°C and at −1°C. The results are shown for α-chymotrypsin in Fig. 2 and for subtilisin in Fig. 3. Generally, a decrease in reaction temperature caused a decrease in the p value. This means that all nucleophiles become more effective at low temperature, and a direct consequence of a low p value is a high peptide yield (see Tables 1 and 2).

The nucleophiles that showed highest efficiency for α-chymotrypsin are the charged amino acids; lysine and arginine. Larger and bulkier hydrophobic amino acids are not that reactive. The specificity order correlates well with previously reported data for immobilized α-chymotrypsin in organic media [16] and for free α-chymotrypsin [13]. The p values obtained for free α-chymotrypsin with Ac–TyrOEt as acyl donor in acetonitrile by Cerovsky and Jakubke (1994) [13] are shown in Fig. 2 for comparison.

The efficiency order for subtilisin is different from the order of α-chymotrypsin. The p values for the various nucleophiles are shown in Fig. 3. Generally, the p values were much higher in subtilisin catalyzed reactions than for those catalyzed by α-chymotrypsin. For the less efficient nucleophiles the p values were in the order of 10 times higher for subtilisin. The lowest p values were reached for small amino acids like glycine, serine and alanine. Hydrophobic amino acid amides were poor nucleophiles and p values up to 400 mM were observed. The specificity for D-alanine was relatively much higher for subtilisin than for α-chymotrypsin. It has earlier been reported that subtilisin shows lower selectivity in the S'1 position compared to α-chymotrypsin for reactions in water [12]. However the p value of D-alanine amide decreased similarly to the p values of the nucleophiles with L-configuration when decreasing the temperature.

The specificity order of the nucleophiles was changed compared to previously reported data for both α-chymotrypsin and subtilisin in water [12]. However, when changing from water to organic media the specificity order will be different [14–16]. In water, the reactivity of α-

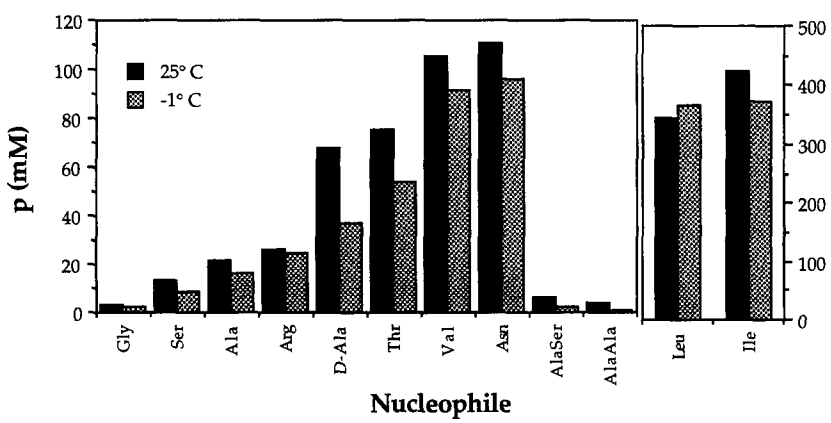

Fig. 3. Nucleophile specificity, p values, for subtilisin Carlsberg at different temperatures. Reaction conditions were as described in Fig. 1, except that 50 mg immobilized subtilisin preparation was used instead of chymotrypsin. The reaction temperatures studied were 25 and −1°C.

chymotrypsin is best correlated with the hydrophobicity of the amino acids, and in organic solvents the normalized Van der Waals volume has the best correlation [16]. Hydrophobic interactions are much less pronounced in organic solvent than in water and these interactions are further decreased when the temperature is decreased [21]. The specificity order of the nucleophiles did not change when decreasing the temperature for the two enzymes. Both α-chymotrypsin and subtilisin showed little preference for the hydrophobic amino acids at the temperatures studied. With the nucleophiles tested in this study, no clear correlation to Van der Waals volume were found.

To confirm the generality of the temperature effect a few dipeptides were tested as nucleophiles. All dipeptides were good nucleophiles for both α-chymotrypsin and subtilisin (Fig. 2 and Fig. 3). The dipeptides have free carboxyl groups which are lacking in the amino acid amides. Compounds with free carboxyl group in water are poor nucleophiles for α-chymotrypsin, but they usually show better reactivity in organic media [10]. The electrostatic repulsion in the active site between the negatively charged carboxyl group and negatively charged residues of α-chymotrypsin seems to be reduced [13]. When decreasing the reaction temperature the dipeptides became even better nucleophiles. The efficiency (p value) decreased by a factor of 2 for all dipeptides except for alanine–arginine for which the decrease was smaller.

3.3. Correlation of the temperature effect on nucleophile specificity with physico-chemical characteristics of the nucleophiles

To pinpoint the temperature effect the ratios of the p value at -1°C and the p value at 25°C were plotted against the Van der Waals volume of the amino acid side chains of the nucleophile (see Tables 1 and 2). A tendency was observed that the temperature effect was more pronounced for nucleophiles with a small Van der Waals volume than for those with a larger (Fig. 4). These results are not surprising because the enzyme molecule probably will become more rigid and less flexible at lower temperatures. A large and bulky amino acid residue will have problems to fit into the active site. The hydrophobicity of the nucleophile is another parameter of interest (see Tables 1 and 2). The temperature effect was studied as a function of the hydrophobicity of the nucleophile. The hydrophobic amino acid amides are the less reactive nucleophiles for both α-chymotrypsin and subtilisin. One reason for this could be that hydrophobic interactions are much less pronounced in an organic solvent and they are even more so at lower temperatures [21]. Therefore the temperature effect showed no dependence of the hydrophobicity of the nucleophile (data not shown). The positive effect of a low temperature on the p value is thus a general effect little affected by the physico-chemical properties of the nucleophile.

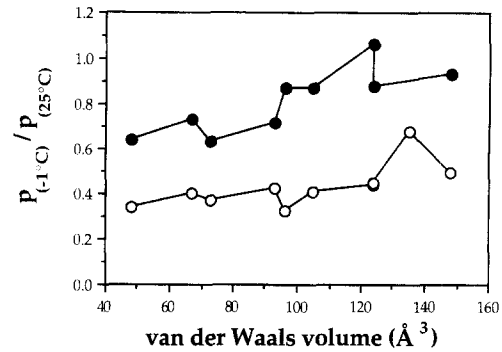

Fig. 4. Correlation of temperature effect with physico-chemical characteristics of the nucleophiles. The effect of Van der Waals volume were studied for two proteases: (○) α-chymotrypsin and (●) subtilisin Carlsberg. The p values were taken from Figs. 2 and 3.

3.4. Influence of temperature on enzyme kinetics

One possible reason for changes in nucleophile specificity is that the reaction scheme is changed. Different reaction schemes are applicable to different peptide synthesis reactions. In all cases for reactions catalyzed by serine proteases there is competition between the nucle-

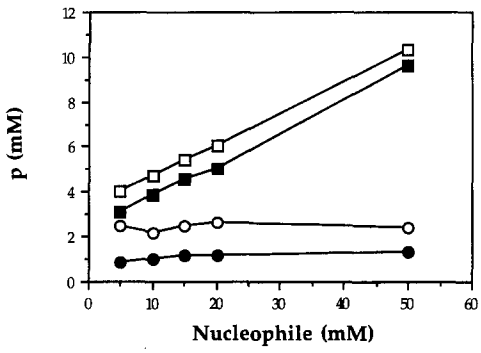

Fig. 5. Effect of nucleophile concentration on p value. The reaction mixture contained 10 mM Ac–PheOEt, 2 to 50 mM of nucleophile, 1% triethylamine, 50 mg immobilized enzyme preparation and acetonitrile with 5% of water. Two nucleophiles were studied, alanine amide (circles) and leucine amide (squares) at 25°C (open symbols) and −1°C (closed symbols).

ophile and water for the acyl enzyme. The first step in the reaction between the nucleophile and the acyl enzyme is normally the formation of a noncovalent complex between the two reactants. In some cases water can react with these complexes thus interfering with the peptide synthesis and in other cases an additional nucleophile molecule can bind to the nucleophile–acyl enzyme complex. Both these modifications of the simplest reaction scheme cause changes in the reaction kinetics.

Ideally the p value is a constant which does not depend on the nucleophile concentration used during the experiment. However, in some cases the kinetics are more complex and the p value varies with the nucleophile concentration [22]. Two nucleophiles with different dependence of p value were studied (Fig. 5). For alanine amide the nucleophile concentration does not affect the p value and this category is the most common for nucleophiles. Leucine amide behave differently from alanine amide. The p value is increasing linearly with the nucleophile concentration. The mechanistic difference between these two categories is that leucine forms an acyl–enzyme–nucleophile complex which is then hydrolyzed and alanine does not form this complex or it is not hydrolyzed [22]. What cannot be ignored is that when the temperature is lowered the concentration dependences are not changed. This indicates that the kinetics for α-chymotrypsin are unaffected by the temperature, and the decrease in p value is a matter of higher nucleophile efficiency compared to that of water.

4. Conclusions

Both subtilisin and α-chymotrypsin catalyzed reactions are positively affected by the temperature. In peptide synthesis reactions the total activity is decreased by a factor of 2.5, but the peptide yield is increased up to 53%, compared to the values obtained at 25°C, on decreasing the temperature from 25 to −1°C. This effect is general for all nucleophiles studied, both those containing only a single amino acid or dipeptides. In multistep synthesis of long peptides it is of great importance to obtain high product yields in all steps. The increase in product yield together with the reduction in by-product formation makes the enzymatic synthesis in organic media at low temperature a powerful tool in practical peptide synthesis.

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References

[1] H.-D. Jakubke, P. Kuhl and A. Könnecke, Angew. Chem., Int. Ed. Eng., 24 (1985) 85.
[2] H. Kise, K. Fujimoto and H. Noritomi, J. Biotechnol., 8 (1988) 279.
[3] P. Clapés, G. Valencia and P. Adlercreutz, Enzyme Microb. Technol., 14 (1992) 575.
[4] K. Nilsson and K. Mosbach, Biotechnol. Bioeng., 26 (1984) 1146.
[5] I. Skuladottir, K. Nilsson and B. Mattiasson, in J Tramper et. al. (Eds.), Biocatalysis in Non-conventional Media, Elsevier, Amsterdam, 1992, p. 307.

[6] S.-T. Chen, S.-Y. Chen, C.-L. Kao and K.-T. Wang, Biotechnol. Lett., 16 (1994) 1075.
[7] Å. Jönsson, P. Adlercreutz and B. Mattiasson, Biotechnol. Bioeng., 46 (1995) 429.
[8] A. Fink, Biochemistry, 12 (1973) 1736.
[9] A. Fink and K. Angelides, Biochemistry, 15 (1976) 5287.
[10] V. Schellenberger, U. Schellenberger, Y. Mitin and H.-D. Jakubke, Eur. J. Biochem., 187 (1990) 163.
[11] P. Clapés and P. Adlercreutz, Biochim. Biophys. Acta, 1118 (1991) 70.
[12] M. Gololobov, T. Voyushina, V. Stepanov and P. Adlercreutz, Biochim. Biophys. Acta, 1160 (1992a) 188.
[13] V. Cerovsky and H.-D. Jakubke, Enzyme Microb. Technol., 16 (1994) 596.
[14] A. Zaks and A. Klibanov, J. Am. Chem. Soc., 108 (1986) 2767.
[15] H. Gaertner and A. Puigserver, Eur. J. Biochem., 181 (1989) 207.
[16] M. Gololobov, T. Voyushina, V. Stepanov and P. Adlercreutz, FEBS Lett., 307 (1992b) 309.
[17] M. Reslow, P. Adlercreutz and B. Mattiasson, Eur. J. Biochem., 172 (1988) 573.
[18] H.-D. Jakubke, in S. Udenfriend and J. Meienhofer (Eds.), The Peptides, Academic Press, London, 1987, p. 103.
[19] H. Kitaguchi, M. Ono, I. Itoh and A. Klibanov, Agric. Biol. Chem., 55 (1991) 3067.
[20] M. Gololobov, E. Kozlova, I. Borisov, U. Schellenberger, V. Schellenberger and H.-D. Jakubke, Biotech. Bioeng., 40 (1992c) 432.
[21] S. Hjertén, J. Rosengren and S. Påhlman, J. Chromatogr, 101 (1974) 281.
[22] M. Gololobov, V. Stepanov, T. Voyushina and P. Adlercreutz, Eur. J. Biochem. 217 (1993) 955.
[23] T. Creighton, Proteins, Structures and Molecular Properties, W.H. Freeman, 2nd ed., 1993, p. 141.
[24] M. Roseman, J. Mol. Biol., 200 (1988) 513.