Boilysin and thermolysin in dipeptide synthesis: a comparative study

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Boilysin (BLN) is an engineered, highly thermostable neutral protease from *Bacillus stearothermophilus*. Its high resistance is based on the stabilization of a surface loop (amino acid residues 55–69) by eight amino acid exchanges, including the introduction of a disulphide bond. In the present study, BLN was compared with the well-known and structurally related thermolysin (from *B. thermoproteolyticus*) with respect to their dipeptide-synthetic properties. The synthesis of the aspartame precursor *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-Asp-Phe-OMe) was used as the model reaction to study its enantioselectivity as well as the influence of neutral salt, temperature and calcium-ion concentration on peptide synthesis. The reactions were performed in homogeneous reaction systems containing DMSO or aliphatic alcohols. Furthermore, the substrate specificity in the synthesis of Z-Asp-X-OMe (X = Phe, D-Phe, Ala, Ile, Leu, Met, Tyr or Val) was examined. The two enzymes showed no difference in time course of reaction by the use of salts as activators or different alcohols as co-solvents. Both enzymes showed a high enantioselectivity towards the amino component in the reaction. They were strongly activated by NaCl, whereas the final product yields were strongly decreased at high NaCl concentrations. Aliphatic alcohols act as an inhibitor, but allow higher product yields compared with purely aqueous medium. Differences between the two enzymes are found at higher temperatures ($\geqslant$ 60 °C) in the absence or at low concentrations of Ca^{2+} ions; BLN proved superior under these conditions, because its stability is less dependent on Ca^{2+} ions. The substrate specificities of BLN and thermolysin at the P_1' position follow the same tendencies, with differences in the initial rates for the conversion of Z-Asp with Ile-OMe, Leu-OMe and Val-OMe.

Introduction

The thermostable neutral metalloprotease thermolysin (TLN; EC 3.4.24.27) from *Bacillus thermoproteolyticus* is well known with respect to its tertiary structure and catalytic properties [1]. Moreover, TLN is one of the most widely used enzymes in peptide synthesis. Thus TLN is exploited for the large-scale industrial production of the aspartame precursor *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-Asp-Phe-OMe). TLN-catalysed synthesis of this and other peptides has been widely studied [2].

In a previous study, a protease of the TLN type with very high thermostability has been created [3]. This enzyme, called boilysin (BLN) [4], is derived from the neutral protease from *B. stearothermophilus* (EC 3.4.24.28), which exhibits 85% sequence identity with TLN, but is less thermostable. Like TLN, this protease contains one Zn^{2+} ion essential for activity and four Ca^{2+} ions with a stabilizing function [5,6]. Extensive mutational studies on the neutral protease from *B. stearothermophilus* have shown that amino acid exchanges in or near the superficial loop region 55–69 influence the thermostability of the molecule more than mutations in other structural regions [7]. The replacement of amino acid residues Ser^{65} and Ala^{69} by Pro [8], the introduction of a salt bridge [9] and the introduction of a disulphide bridge [10] resulted in a dramatic improvement of thermal stability. In BLN, eight of these stabilizing amino acid substitutions ($Ala^4 \rightarrow$ Thr, A4T; $Gly^8 \rightarrow$ Cys, G8C; $Thr^{56} \rightarrow$ Ala, T56A; $Gly^{58} \rightarrow$ Ala, G58A; $Asn^{60} \rightarrow$ Cys, N60C; $Thr^{63} \rightarrow$ Phe, T63F; $Ser^{65} \rightarrow$ Pro, S65P; $Ala^{69} \rightarrow$ Pro, A69P) are combined, which make the enzyme resistant to boiling. Its half-life is 170 min at 100 °C, whereas the half-life of TLN and the wild-type (WT) enzyme from *B. stearothermophilus* are 1 and < 0.5 min respectively [3]. Despite the extreme stabilization, the activity of BLN towards casein or the synthetic peptide substrates 3-(2-furylacryloyl)-L-glycyl-L-leucinamide and 3-(2-furylacryloyl)-L-alanyl-L-phenylalaninamide is not significantly changed compared with the WT [3]. In contrast with naturally occurring thermophilic enzymes, which are often

Key words: aspartame, *Bacillus stearothermophilus*, neutral protease, organic solvent, P_1' specificity.

Abbreviations used: BLN, boilysin; OMe, methyl ester; TFA, trifluoroacetic acid; TLN, thermolysin; WT, wild-type; Z, *N*-(benzyloxycarbonyl).

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less active than their mesophilic counterparts at lower temperatures, the activities of the natural protease from *B. stearothermophilus* and BLN are very similar at temperatures below 70 °C. At higher temperatures, the activity of BLN increases further, whereas the activity of the WT protease decreases because of thermal inactivation [3]. In the hydrolysis of food proteins, BLN showed the highest level compared with three industrial proteases [11].

Owing to the excellent stability properties of BLN, the question arises whether this enzyme is also appropriate for peptide synthesis. Therefore the aim of the present study was to compare the synthetic potential of TLN and BLN. The synthesis of Z-Asp-Phe-OMe from Z-Asp and Phe-OMe was used as the model reaction to study the influence of different reaction parameters on the initial rates as well as on product yields. The comparison was further extended to the synthesis of dipeptides of a related type, Z-Asp-X-OMe (X = Phe, D-Phe, Ala, Ile, Leu, Met, Tyr or Val). In addition to BLN and TLN, a Cys-free mutant ($Cys^{288} \rightarrow Leu$) [12] of the neutral protease from *B. stearothermophilus* was included in the present study. This enzyme is nearly identical with the WT in its activity and stability and was used as WT in this study.

If not stated otherwise, amino acids are in L-configuration.

Materials and methods

Materials

TLN was purchased from Sigma and used without further purification. Z-Asp · HCl, Phe-OMe · HCl, D-Phe-OMe · HCl, Ala-OMe · HCl, Ile-OMe · HCl, Leu-OMe · HCl, Met-OMe · HCl, Tyr-OMe · HCl and Val-OMe · HCl were obtained from Bachem (Heidelberg, Germany). Z-Asp-Phe-OMe was enzymically synthesized from Z-Asp and Phe-OMe [13]. All other chemicals were of analytical grade and purchased from Sigma, Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

Enzyme production and purification

The protease-deficient strain *B. subtilis* DB117 [14] was used as the host for plasmid pGE530 with mutation $Cys^{288} \rightarrow Leu$ (C288L) [12] or for plasmid pGE501 with mutations A4T/G8C/T56A/G58A/N60C/T63F/S65F/A69P [3]. Production and purification of WT and BLN were performed as described previously [14] using affinity chromatography on Bacitracin–silica [15]. The electrophoretically homogeneous enzymes were stored in elution buffer (pH 5.3), containing 20 mM sodium acetate, 5 mM $CaCl_2$, 2.5 M NaCl, 20% (v/v) propan-2-ol and 0.03% (w/v) sodium azide.

Protein determination

Protein concentration was determined with bicinchoninic acid protein assay reagent (Pierce, Rockford, U.S.A.) using BSA as a standard.

Synthesis of Z-Asp-X-OMe

Standard synthesis of Z-Asp-X-OMe was performed at 22 °C (room temperature) in 50 mM Mes buffer (pH 6.2), containing 5 mM $CaCl_2$ and 30% (v/v) DMSO in a final volume of 200 μl. The initial concentrations of the substrates Z-Asp and X-OMe were 20 and 80 mM respectively. The protein concentrations of TLN, WT and BLN were 100 μg/ml each. After various time periods (0–200 h), samples of 10 μl were withdrawn. The reaction was stopped by adding 10 μl of 0.3% (v/v) trifluoroacetic acid (TFA). Before HPLC analysis, samples were diluted with water to a volume of 100 μl.

In our studies to determine the influence of NaCl on the synthesis of Z-Asp-Phe-OMe, the concentration of DMSO was only 10% (v/v).

To examine the effect of Ca^{2+} ions on the synthesis of Z-Asp-Phe-OMe at different temperatures, the reaction buffer contained 0–20 mM $CaCl_2$. Before addition to the reaction mixtures, the enzymes were dialysed against 50 mM Mes buffer (pH 6.2) to remove the Ca^{2+} ions from the enzyme preparation.

In the experiments with different alcohols, DMSO was replaced by 45% (v/v) methanol, ethanol, propan-1-ol, propan-2-ol or glycerol.

Results shown in the Figures are means of double determinations and the data in Table 1 are from triple determinations.

HPLC analysis

Reaction mixtures were analysed by HPLC (Merck-Hitachi, Tokyo, Japan) using a nucleosil C_8 (5 μm particle size) column (Merck). Z-Asp-X-OMe, Z-Asp and X-OMe were separated by a two-step procedure using mixtures of solvent A [acetonitrile containing 0.07% (v/v) TFA] and solvent B [water containing 0.1% (v/v) TFA]. Solvent A/solvent B in the ratio 1:3 was applied in the first step (0–2 min) and the ratio of the solvents was 2:3 for the second step (2–6 min). The flow-rate was 2 ml/min. Peaks were detected at 254 nm. The product Z-Asp-Phe-OMe was quantified from the ratio of its peak area to the sum of the peak areas of Z-Asp and Z-Asp-Phe-OMe. In the case of synthesis with other amino components, the amount of the products was determined by the decrease in peak area corresponding to Z-Asp. The concentrations of Z-Asp and Z-Asp-Phe-OMe were calculated from standard curves. Concentrations of the standard solutions were determined by the molar absorbance coefficients of Z-Asp (208 $M^{-1} \cdot$

Table 1 Substrate specificity in P_1' position for TLN, BLN and WT

Z-Asp (20 mM) and amino component (X-OMe) (80 mM) were converted at 22 °C in 50 mM Mes (pH 6.2), containing 5 mM $CaCl_2$, 30% (v/v) DMSO and 100 μg/ml enzyme. b.d., below detection limit.

Amino component	Initial rate (nmol/min)		
	WT	BLN	TLN
Ile-Ome	0.80±0.03	0.82±0.07	1.81±0.20
Phe-Ome	0.84±0.14	0.98±0.21	0.95±0.19
Leu-Ome	0.070±0.020	0.096±0.014	0.34±0.03
Val-OMe	0.032±0.002	0.032±0.001	0.054±0.009
Met-OMe	0.026±0.012	0.028±0.012	0.018±0.006
Tyr-OMe	0.004±0.001	0.004±0.001	0.004±0.001
Ala-OMe	b.d.	b.d.	b.d.

cm^{-1}) and Z-Asp-Phe-OMe (387 $M^{-1} \cdot cm^{-1}$) at 257 nm in 40 mM Tris/HCl (pH 7.4) containing 10 mM $CaCl_2$ at room temperature [16].

Results and discussion

Reaction systems

To compare peptide synthesis by BLN and WT with that of TLN, we used the well-known reaction between Z-Asp and Phe-OMe, yielding the aspartame precursor Z-Asp-Phe-OMe. The initial reaction rates as well as product yields were considered at different ratios of substrate enantiomers, concentrations of neutral salt and calcium ions or temperatures. These studies were performed in a homogeneous system containing buffer and a water-miscible organic solvent. In the standard reaction mixture, 30% (v/v) DMSO in 50 mM Mes buffer (pH 6.2) was used. These conditions are not optimum with respect to product synthesis, but they allow a good comparison of the three enzymes without solubility problems or chemical side reactions. BLN tends to aggregate at protein concentrations $\geqslant$ 250 μg/ml in pure buffer; at 30% DMSO, 100 μg/ml can be used. The initial rates increase linearly with the increasing enzyme concentration over the range 0–200 μg/ml (results not shown). Figure 1 demonstrates the reaction progress in the absence and presence of DMSO. In this system, there are no significant differences for the three enzymes. In all these cases, the initial rates are higher in the absence of DMSO, whereas higher final yields are reached in the presence of organic solvent, demonstrating a shift in the product concentrations in equilibrium.

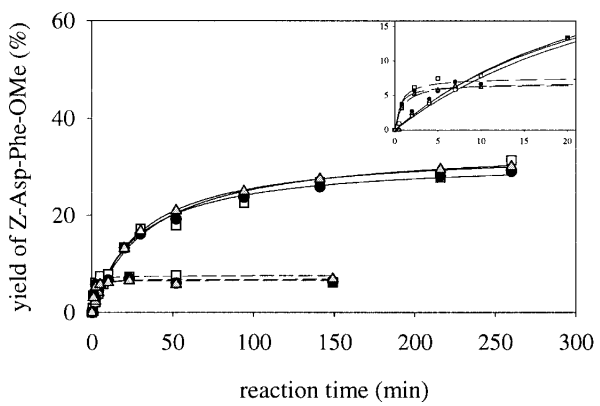

Figure 1 Time course of synthesis of Z-Asp-Phe-OMe catalysed by BLN (●), TLN (□) and WT (▲) in the presence (—) and absence (- -) of 30% (v/v) DMSO at 22 °C

The reaction mixtures contained 20 mM Z-Asp, 80 mM Phe-OMe and 100 μg/ml enzyme in 50 mM Mes buffer (pH 6.2) containing 5 mM $CaCl_2$. The inset shows the initial phase of the reaction magnified.

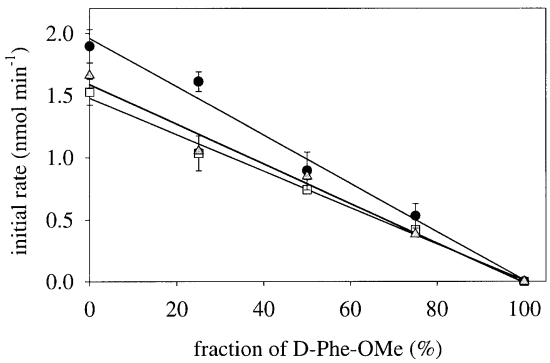

Figure 2 Effect of D-Phe-OMe on the initial rate of synthesis of Z-Asp-Phe-OMe catalysed by BLN (●), TLN (□) and WT (▲)

Z-Asp (20 mM) and Phe-OMe+D-Phe-OMe (80 mM) were converted at 22 °C in 50 mM Mes buffer (pH 6.2) containing 5 mM $CaCl_2$, 30% (v/v) DMSO and 100 μg/ml enzyme.

Enantioselectivity

In the standard reaction system, Phe-OMe was replaced by different percentages of D-Phe-OMe, whereas the total amount of the ester was kept constant. With all three enzymes, no reaction was found with the pure D-enantiomer (Figure 2), which indicates a strict stereoselectivity for the L-enantiomer by BLN, WT and TLN. The initial rate linearly decreases with the fraction of D-Phe-OMe for all the three enzymes (Figure 2), showing that the reaction rate depends on the concentration of L-Phe-OMe only and the presence of the D-enantiomer neither enhances nor inhibits the reaction. Therefore TLN, WT and BLN do not differ with respect to enantioselectivity. The results for TLN are consistent with those described previously [17].

Influence of NaCl

Since high concentrations of neutral salts are known to activate TLN in hydrolysis as well as in synthesis [16], the influence of NaCl on the time course of Z-Asp-Phe-OMe synthesis catalysed by BLN, WT and TLN were compared. Figure 3(A) shows the effect of 0–2.4 M NaCl on the initial rate, whereas Figure 3(B) shows the product yield at equilibrium. For all the three enzymes, an exponential enhancement of the initial rate with increasing NaCl

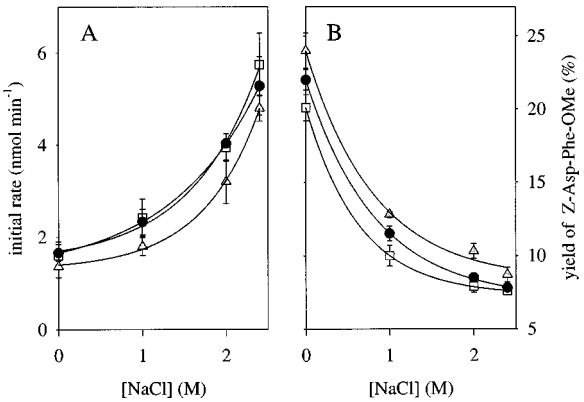

Figure 3 Effect of NaCl on (A) initial rate and (B) yield of synthesis of Z-Asp-Phe-OMe catalysed by BLN (●), TLN (□) and WT (▲)

Z-Asp (20 mM) and Phe-OMe (80 mM) were converted at 22 °C in 50 mM Mes buffer (pH 6.2), containing 5 mM $CaCl_2$, 30% (v/v) DMSO and 100 μg/ml enzyme.

concentration was observed, whereas the product yield decreased in a similar manner. In the presence of 2.4 M NaCl, the enzyme activity was 3.5 times higher than in the absence of salt, whereas the yield of synthesis was approx. three times lower. Because an exponential correlation between the absorbance of TLN and the concentration of NaCl was observed [16], the effect of salt activation might be caused by conformational changes in the enzymes. Another reason for this effect can be seen in changes of the dielectric constant of the medium [18]. The total correspondence of the activation effects among the three enzymes shows the great similarity between TLN and the variants derived from the neutral protease from *B. stearothermophilus* on the one hand, and the negligible influence of the eight mutations in BLN on catalytic properties on the other. The opposite effect of NaCl on the product yields is probably due to the influence of the salt ions on the activity coefficients of the reaction components, thereby changing their equilibrium concentrations. The alternative interpretation that enzymes may be inactivated by the high salt concentrations can be excluded, because NaCl is known to increase stability of TLN [19].

Influence of temperature and calcium ions

TLN, WT or BLN remarkably differ in thermostability (BLN > TLN > WT) and in the influence of Ca^{2+} ions on the stability, which should be reflected more in product yields of peptide synthesis than in initial rates. As expected, initial rates of Z-Asp-Phe-OMe synthesis increased with increasing temperature but were in the same range for all three enzymes (6, 9 and 11 nmol/min at 40, 60 and 70 °C respectively). In contrast, final product yields achieved by the different enzymes showed differences in their dependence on temperature and Ca^{2+} ion concentration (Figure 4). As

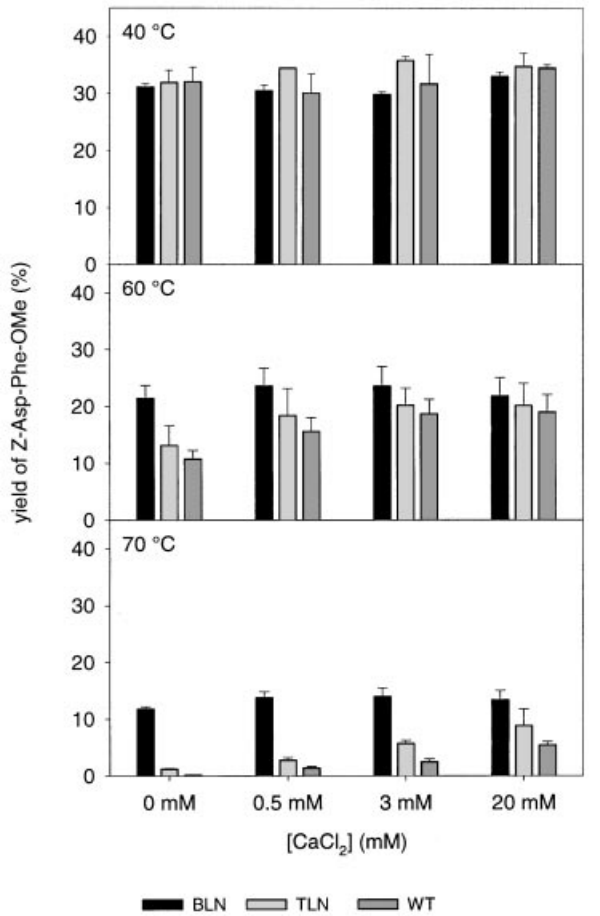

Figure 4 Effect of Ca^{2+} ions and temperature on the yield of Z-Asp-Phe-OMe

Z-Asp (20 mM) and Phe-OMe (80 mM) were converted in 50 mM Mes buffer (pH 6.2) containing 0, 0.5, 3 or 20 mM $CaCl_2$, 30% (v/v) DMSO and 100 μg/ml enzyme at 40, 60 or 70 °C. The final yields of synthesis were reached after 250 h at 40 °C, 100 h at 60 °C and 50 h at 70 °C.

revealed by HPLC, the generally decreased product yields at higher temperatures are due to the side reactions. With increasing temperature, an increasing fraction of the substrate Phe-OMe is non-enzymically converted into Phe-OH, which is much less reactive than the ester form. Therefore product yields can be compared only at the same temperature. Differences in the product yields achieved at the same temperature reflect the different thermostabilities of the three enzymes and their dependence on calcium ions. Although the amount of products formed by the three enzymes were the same at 40 °C, there were distinct differences at 70 °C, which decreased with increasing Ca^{2+} ion concentrations. At 70 °C, the product yields increase in the order WT < TLN < BLN. Obviously, the maximum product concentrations are not reached with TLN or WT at this temperature, even if the stability and, therefore, the product yields, are higher at increased Ca^{2+} ion concentra-

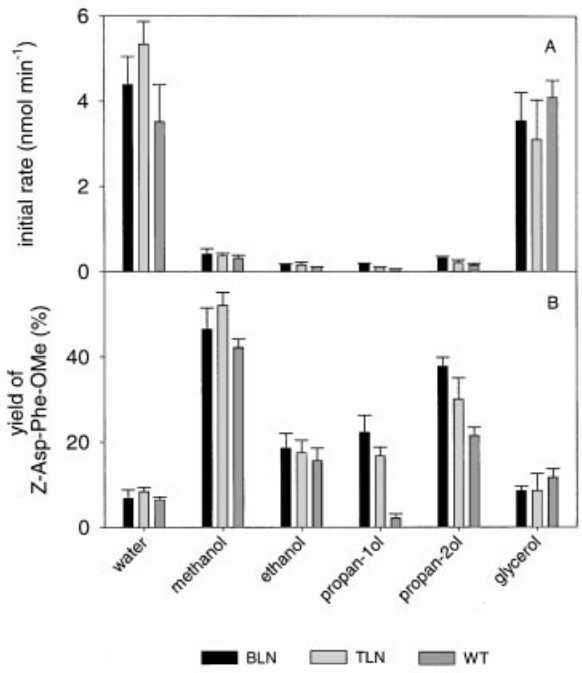

Figure 5 Effect of various alcohols on the enzymic synthesis of Z-Asp-Phe-OMe

(**A**) Initial rates. (**B**) Product yields after 200 h. Z-Asp (20 mM) and Phe-OMe (80 mM) were converted at 22 °C in 50 mM Mes buffer (pH 6.2), containing 5 mM $CaCl_2$, 100 μg/ml enzyme with or without 45% (v/v) of either methanol, ethanol, propan-1-ol, propan-2-ol or glycerol. In the case of synthesis in methanol, ethanol, propan-1-ol and propan-2-ol, the equilibrium yields were not reached until after 200 h.

tions. The differences in enzyme stability emerge also at 60 °C, particularly in the absence of stabilizing Ca^{2+} ions, but they are less pronounced. The results at 60 and 70 °C demonstrate that the stabilizing effect of Ca^{2+} ions is strongest for WT, less for TLN and insignificant for BLN. According to mutation experiments and computer modelling, these differences can be attributed to different binding affinities at the Ca^{2+}-binding site in the critical surface loop of the protein around residues 55–69 [6].

Effect of aliphatic alcohols

To study the effect of different alcohols on dipeptide synthesis, the time courses of Z-Asp-Phe-OMe synthesis were investigated in aqueous mixtures with methanol, ethanol, propan-1-ol, propan-2-ol and glycerol. Figure 5 represents the initial rates and product yields of the reaction in 45% (v/v) alcohol at room temperature, including the results obtained in buffer solution without any organic solvent. TLN, WT and BLN reveal a very similar behaviour in the initial rates (Figure 5A). All univalent alcohols act as inhibitors of the proteases, whereas the reaction rate in glycerol is similar to that in water. The inhibitory effect of univalent alcohols on both TLN and neutral protease from *B. stearothermophilus* is well known [18] and it is used as a protection against auto-proteolysis during storage. By soaking TLN crystals in 2–100% propan-2-ol, the inhibitory effect could be clearly attributed to the occupation of the main subsites in the active site of TLN [20]. However, in the case of the product yields after 200 h reaction time, some differences between the three enzymes can be observed (Figure 5B). BLN, TLN and WT behave similarly in pure buffer or mixtures with methanol, ethanol and glycerol. The univalent polar solvents methanol and ethanol give 4- and 2-fold higher yield respectively when compared with the reaction in buffer, which reflects the higher product concentrations after long reaction periods. The maximum yield of synthesis in glycerol is similar to that in pure buffer, although glycerol has no dramatic effect on the reaction rate and is a solvent with well-documented protein stabilization effects [21]. As derived from the HPLC chromatograms, the low yields in glycerol are caused by the rapid hydrolysis of the substrate ester Phe-OMe to Phe-OH, which is a less reactive nucleophile as mentioned above.

In 45% (v/v) propan-1-ol and propan-2-ol, TLN and BLN yield similar results, whereas WT shows lower yields, especially for the synthesis in the least polar solvent propan-1-ol. In this case, the yield is lower than in pure aqueous solution. This result can be attributed to solvent denaturation of WT. Because thermostability mostly corresponds to solvent stability [22,23], WT is expected to be less resistant towards organic solvents when compared with TLN and BLN.

Substrate specificity at the P_1' position

The substrate specificities of BLN, WT and TLN in dipeptide synthesis were compared with respect to amino acid residues in P_1' position. In hydrolysis, TLN and the neutral protease from *B. stearothermophilus* are known to prefer bulky hydrophobic amino acids [23,24]. The same reaction conditions as for the synthesis of Z-Asp-Phe-OMe were used, but the amino component Phe-OMe was replaced with Ala-OMe, Ile-OMe, Leu-OMe, Met-OMe, Tyr-OMe, Phe-OMe or Val-OMe; Table 1 summarizes the initial rates for the corresponding reactions. TLN, WT and BLN show nearly the same order of substrate preference: Ile-OMe (TLN) or Phe-OMe (BLN, WT) > Leu-OMe > Val-OMe > Met-OMe > Tyr-OMe. No dipeptide product was formed with Ala-OMe as amino component. With WT and BLN, the initial rates for all reactions were identical within the range of error. Since both enzyme variants are derived from the neutral protease from *B. stearothermophilus* and do not differ in their active sites, this result is not surprising, but confirms that extensive mutations are possible without changes of the substrate specificity. Also, the initial rates for

TLN show no significant differences with Phe-OMe, Tyr-OMe and Met-OMe in comparison with the initial rates for BLN and WT, whereas the initial rates with Leu-OMe, Ile-OMe and Val-OMe are significantly higher for TLN. This difference can be explained by a different amino acid in the S_1'-subsite region (Leu^{133} in TLN and Phe^{133} in WT and BLN). The large Phe^{133} residue might influence the binding of the bulky amino acids Ile, Leu and Val. Thus the mutation $Phe^{133} \rightarrow Leu$ in neutral protease from *B. stearothermophilus* was shown to convert the hydrolytic activity towards dipeptide and tripeptide substrates into the more TLN-like characteristics [25].

Conclusions

Comparison of BLN, which is characterized by a very high thermostability, and TLN, which is approved as a catalyst for enzymic peptide synthesis, showed that both enzymes behave very similarly in peptide synthesis. As demonstrated for the synthesis of Z-Asp-Phe-OMe, both enzymes show a strong enantioselectivity. They are highly activated by neutral salts, whereas the product yields in equilibrium show the opposite tendency. They are inhibited by univalent aliphatic alcohols, but the equilibrium product yields are higher in the presence of high proportions of alcohols. In the reaction of Z-Asp with different amino components, the enzymes show nearly the same order of amino acid preferences with distinct differences of the reaction rates for some amino acids (Ile, Leu and Val). Differences between BLN and TLN emerge at higher temperatures in the absence or at low concentrations of Ca^{2+} ions, where higher reaction yields are achieved due to higher stability of BLN. Therefore BLN could be an interesting alternative for TLN in all reactions where the presence of Ca^{2+} ions is disturbing. Since Ca^{2+} ions very often interfere with downstream processing, this enzyme may be important for several applications.

Acknowledgments

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