Journal of Peptide Science
J. Peptide Sci. **6**: 541–549 (2000)

The Applicability of Subtilisin *Carlsberg* in Peptide Synthesis

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Received 5 January 2000
Accepted 2 March 2000

Abstract: The synthesis of peptide bonds catalysed by subtilisin *Carlsberg* was studied in different hydrophilic organic solvents with variable H_2O concentration. Z-Val-Trp-OMe and Z-Ala-Phe-OMe were used as acyl donors, and a series of amino acid derivatives, di- and tripeptides of the general structure Xaa-Gly, Gly-Xaa, Gly-Gly-Xaa (Xaa represents all natural L-amino acids except cysteine) and other peptides were used as nucleophiles. A comparative study of the enzymatic synthesis in aqueous DMF (50%, v/v) and acetonitrile containing 10% (v/v) of H_2O demonstrated that the yields of peptide products were higher in most cases when acetonitrile with low H_2O concentration was used. The acylation of weak nucleophiles was improved in organic solvents with very low H_2O concentration (2%). The reactions in anhydrous Bu^t-OH proceeded with substantially lower velocity. Generally, the restricted nucleophile specificity of the enzyme for glycine and hydrophilic amino acid residues in P1′ position, as well as numerous side reactions, limit the utilization of subtilisin in peptide synthesis, especially in the case of the segment condensations. Contrary to the published data, we have proved that proline derivatives were not acylated in any media with the help of subtilisin *Carlsberg*. Effective ester hydrolysis of a protected nonapeptide corresponding to the *N*-terminal sequence of dicarba-eel-calcitonin catalysed by sublilisin was achieved. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: enzymatic peptide synthesis; subtilisin; nucleophile specificity; proline

INTRODUCTION

Proteases are becoming increasingly popular as catalysts in peptide chemistry, namely for peptide bond formation [1]. In a kinetically controlled synthesis, the aminolysis/hydrolysis ratio is the key parameter for obtaining high peptide yield [2]. To promote the aminolysis over the ester hydrolysis, utilization of efficient nucleophiles or working in low H_2O media is often desirable. Possibilities of using proteases in enzymatic peptide synthesis in non-aqueous solvents as a means of improving yields have been studied extensively [3–5]. With respect to the stability of proteolytic enzymes in organic media subtilisins, they are considered to be the most efficient enzymes among the family of proteases used for this purpose [6–8]. Subtilisins are bacterial serine proteases, classified today as subtilisin BPN′ or subtilisin *Carlsberg*, and are marketed by several companies. These two species have very similar three-dimensional structures, but differ in their primary structure, which results in different catalytic activities [9,10]. Several other proteases of the subtilisin group with different properties have been reported in the literature [10]. The nomenclature of these enzymes, which has been changed several times, and various labelling of commercial samples of subtilisins has caused confusion in the studies [10–12]. In contrast to the well-known substrate specificity of these enzymes, the nucleophile

Abbreviations: OMe, methyl ester; OBzl, benzyl ester.

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specificity [2] in acyl transfer reactions has not been sufficiently investigated. The nucleophile specificity of subtilisin BPN′ in acetonitrile with low H_2O content has been systematically studied in our laboratory [12]. The values of partition constants estimated in the acyl transfer reaction for a large series of nucleophiles indicate preference for glycine and small hydrophilic amino acid residues in P1′ position (binding site notation according to Schechter and Berger [13]), which is principally in agreement with the majority of published data [14–16]. The proline residue in this position was not acylated [12]. A few papers also describe the acylation of bulky hydrophobic amino acid derivatives and dipeptides with high yields, using different samples of subtilisins [16–18]. The peptide synthesis in a high yield, using proline derivatives as nucleophiles catalysed by alcalase or subtilisin *Carlsberg* in Bu^t-OH published by Chen, was a great surprise to us [19]. This finding led us to extensively investigate enzymatic peptide synthesis catalysed by subtilisin *Carlsberg*, with respect to its nucleophile specificity in various media. Special attention has been paid to the acylation of proline derivatives and Pro containing peptides, in order to either confirm or to contradict Chen's results [19]. The measure of nucleophile efficiency of various amino components in this study is expressed in terms of HPLC-yields of peptide products in the acyl transfer reaction, avoiding the very laborious evaluation of partition constants [12]. In the course of the synthesis of the dicarba analog of eel calcitonin [20], we made use of the high esterase activity of subtilisin *Carlsberg* for the hydrolysis of its *N*-terminal protected nonapeptide methyl ester cyclo[1-6]-Ser(Bu^t)-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Asu-Val-Leu-OMe. We wanted to avoid the side reactions that could be expected if the asparagine containing peptide was subjected to alkaline hydrolysis.

MATERIALS AND METHODS

Materials

Subtilisin *Carlsberg* (Proteinase, bacterial; 15.4 U/mg), Cat. No. 82528, was from FLUKA BioChemika, (Switzerland). We verified the identity of this sample by amino acid analysis, which was consistent with the data in the literature [9]. Z-Val-Trp-OMe, Z-Ala-Phe-OMe and the majority of nucleophilic components were purchased from BACHEM (Bubendorf, Switzerland), others from SIGMA (USA). Deamino-dicarba-eel-calcitonin sequence 1-9; cyclo[1-6]Ser(Bu^t)-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Asu-Val-Leu-OMe was synthesized in solution, as described [20]. The peptide had 80% HPLC purity, with a satisfactory amino acid composition; FAB-MS: $m/z = 1068.4$ $[M^+ + H]$.

HPLC Analyses

HPLC analyses were performed using a Thermo Separation Products instrument (USA), equipped with a Spectra Series P 100 pump, UV 100 detector, Chrom Jet integrator, and a Merck 25×0.4 cm column, packed with LiChrospher WP-300 (RP-18, 5 μm). Samples were analysed isocratically, with various mixtures of methanol (50–60% v/v) in 0.1% aqueous trifluoroacetic acid, at a flow rate of 1 ml/min. The reactions with Z-Val-Trp-OMe and Z-Ala-Phe-OMe were monitored at 280 nm and 254 nm, respectively. We assumed that the extinction coefficients of the hydrolysis products, and the synthesis products in acyl transfer reaction were equal [12]. For the Tyr-, Trp- and Phe-containing nucleophiles, the ε of the peptide products were considered as a sum of the ε of the Z-Val-Trp-OH (Z-Ala-Phe-OH) and the nucleophile. Certain peptide products were isolated by gradient HPLC with 0.1% TFA/methanol system, at a flow rate of 3 ml/min on 25×1 cm Vydac C18 column.

Enzymatic Peptide Synthesis

Reactions of Z-Val-Trp-OMe with nucleophiles in aqueous dimethylformamide. The reactions were carried out at room temperature in 200 μl of a DMF/0.2 M sodium veronal, pH 9.5 (1/1) mixture. Stock solution of the acyl donor Z-Val-Trp-OMe (0.1 M) was prepared in DMF and stored at −15°C. The nucleophile (20 μmol) was dissolved in 0.2 M sodium veronal, pH 9.5 (98 μl), mixed with the stock solution of acyl donor (100 μl, 10 μmol), neutralized with piperidine (2 or 4 μl), and the reaction was started by adding 2 μl of the enzyme stock solution (10 mg/100 μl) in H_2O. The time course of the reactions was followed by HPLC analysis. After 1 h of reaction, the mixtures were diluted by 1.5 ml of the stop solution of 50% aqueous methanol, containing 1% trifluoroacetic acid, and analysed by HPLC for final yield estimations.

Reactions of Z-Val-Trp-OMe with nucleophiles in acetonitrile containing 10% (v/v) of H_2O. The reactions were carried out at room temperature in 200 μl of an acetonitrile/DMF/H_2O (8/1/1) mixture for 24–48

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h. Stock solution of the acyl donor Z-Val-Trp-OMe (0.1 M) was prepared in acetonitrile/DMF (4/1) mixture, and stored at −15°C. The nucleophile (20 μmol) was suspended in acetonitrile (80 μl), and H_2O (16 μl) mixed with the stock solution of the acyl donor (100 μl, 10 μmol), neutralized with piperidine, (2 or 4 μl), and the reactions were started by adding 4 μl of the enzyme stock solution (10 mg/100 μl) in H_2O. The reactions were analysed as described above.

Reactions of Z-Ala-Phe-OMe with nucleophiles in various media. The reactions were carried out at room temperature in 100 μl of the total volume in the following mixtures: DMF/0.2 M sodium veronal, pH 9.5 (1/1), acetonitrile/H_2O (9/1), acetonitrile/H_2O (49/1), Bu^t-OH/H_2O (49/1) and Bu^t-OH for the time indicated in Table 2. The concentrations of the acyl donor and nucleophiles were 0.05 M and 0.2 M, respectively. The reactions were started after neutralization of nucleophile hydrochloride with piperidine (2 μl), by the addition of subtilisin (0.05–0.5 mg) in H_2O (2 μl) or solid enzyme (0.5 mg), and followed by HPLC.

An attempt to reproduce Z-Ala-Phe-Pro-NH_2 synthesis. The synthesis was exactly reproduced according to Chen's paper [19] on a ten times smaller scale. A mixture of Z-Ala-Phe-OMe (384 mg, 1 mmol) Pro-NH_2 (339 mg, 3 mmol) and subtilisin *Carlsberg* (25 mg) in Bu^t-OH (2 ml) was stirred at 25°C for 48 h. The HPLC analysis (254 nm) of the mixture shows only the presence of Z-Ala-Phe-OMe, and a trace of Z-Ala-Phe-OH. No tripeptide was detected. Nevertheless, we followed the work up procedure, using ethyl acetate extraction, as described [19]. The crude isolated 'product' was characterized by amino acid analysis and FAB-MS as Z-Ala-Phe-OMe. In another experiment, the reaction mixture was concentrated in vacuum, the residue was dried and analysed by FAB-MS. No molecular peak of the tripeptide was detected.

Unsuccessful synthesis of Z-Ala-Phe-Pro-OBzl. Triethylamine (22.5 μl, 0.16 mmol) was added to a suspension of Pro-OBzl HCl (36.3 mg, 0.15 mmol) in Bu^t-OH (150 μl). After the addition of Z-Ala-Phe-OMe (38.4 mg, 0.1 mmol), H_2O (3 μl) and subtilisin *Carlsberg* (2.5 mg), the mixture was stirred at 25°C. The time course of the reaction followed by HPLC shows, after 4 days, only the formation of Z-Ala-Phe-OH (20%), without any indication of tripeptide synthesis.

Enzymatic hydrolysis of the methyl ester of dicarba-eel-calcitonin sequence 1–9. Subtilisin (0.5 mg) was added to a suspension of cyclo[1-6]-Ser(Bu^t)-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Asu-Val-Leu-OMe (50 mg), in a mixture of methanol (0.5 ml) and 0.2 M carbonate-bicarbonate buffer, pH 9 (0.5 ml). The mixture was vigorously stirred at room temperature for 40 min, when the suspension cleared up, and the HPLC indicated complete conversion of the ester. The product was purified by filtration through a column of Dowex 50 (1 ml) in 50% aqueous methanol. The eluate was concentrated, lyophilized and the residue was triturated with n-hexane, affording 35 mg (71%) of the peptide with corresponding amino acid analysis; FAB-MS: $m/z = 1054[M^+ + H]$.

RESULTS AND DISCUSSION

Reactions of Z-Val-Trp-OMe with Various Nucleophiles in Aqueous Medium

The subtilisin-catalysed peptide bond formation between Z-Val-Trp-OMe and various nucleophilic components was studied in an aqueous system of sodium veronal, pH 9.5, containing 50% (v/v) DMF, necessary for the solubilization of the acyl donor. The presence of a strong organic base (piperidine), equal to the acidic groups of amino component, ensured that its α-amino group was in the unprotonated form. The competitive ester hydrolysis in all tested reactions indicated that the enzyme retained its activity until the acyl donor had completely reacted. Under these conditions, we had not observed secondary hydrolysis [2] within a prolonged reaction period. The results of reactions with Xaa-NH_2, Xaa-Gly, Gly-Xaa, and Gly-Gly-Xaa peptides are shown in Figures 1–4, respectively. The situation concerning some Ala containing dipeptides is demonstrated in Figure 5. The yields of synthesized peptide amides dropped dramatically when a side chain was present in any of the amino acid amides tested as nucleophiles (Figure 1), as compared with glycine amide. Only the residues with small side chains were incorporated into peptides, to a certain degree. Evidently, there was no significant electrostatic interaction of the charged amino acid residues with the S1′ subsite [21,22]. Uncharged Asn-NH_2 and Gln-NH_2 were not acylated significantly better than Asp(Glu)-amides. Reaction with Lys-NH_2 gave slightly higher yields of the product owing to the extra acylation of the ε-amino group, resulting in two peptide regioisomers [23]. No acylation of Pro-NH_2 was observed, besides concurrent ester hydrolysis of the acyl component. The same trend of nucleophile specificity was evident for the peptides of the Xaa-Gly series (Figure 2). However, by

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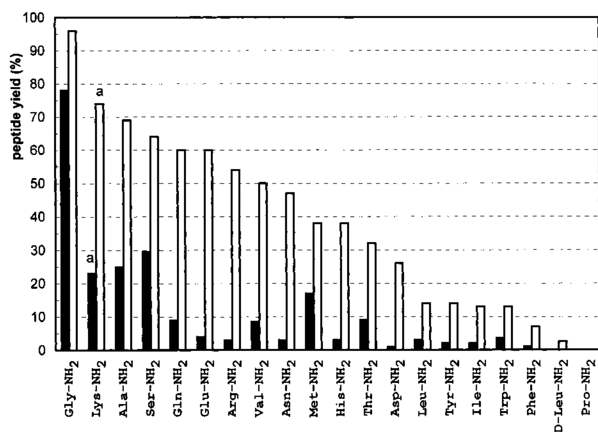

Figure 1 Peptide syntheses catalysed by subtilisin *Carlsberg*, using Z-Val-Trp-OMe as acyl donor, and a series of amino acid amides as nucleophiles in DMF/0.2 M sodium veronal, pH 9.5 (1/1) ■ and acetonitrile/DMF/H_2O (8/1/1) □. (a) Yield represents the sum for the formation of α- and ε-isomers.

substituting the amide group by a glycine residue, a further drop of the yield of peptide products was apparent for all amino acid residues, except glycine. The side chain of a simple amino acid amide is fairly relaxed, and can, therefore, interact within the S1′ subsite as it is a building block of a peptide chain. We observed the same effect when we investigated nucleophile specificity of α-chymotrypsin [24]. It was previously concluded that the S1′ subsite of subtilisin BPN′ requires for catalysis the assistance of the S2′ subsite which prefers hydrophobic bulky

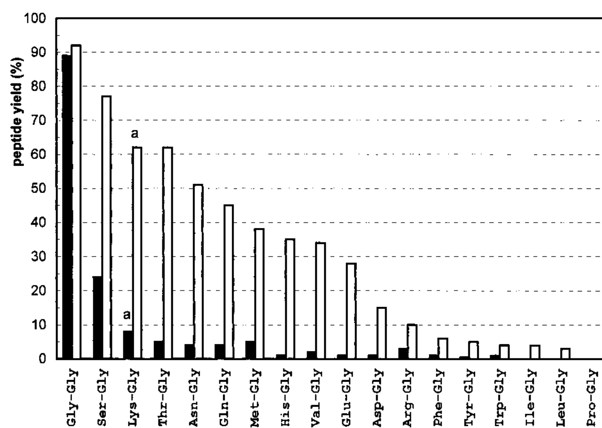

Figure 2 Peptide syntheses catalysed by subtilisin *Carlsberg*, using Z-Val-Trp-OMe as acyl donor, and a series of Xaa-Gly dipeptides as nucleophiles in DMF/0.2 M sodium veronal, pH 9.5 (1/1) ■ and acetonitrile/DMF/H_2O (8/1/1) □. (a) Yield represents the sum for the formation of α- and ε-isomers.

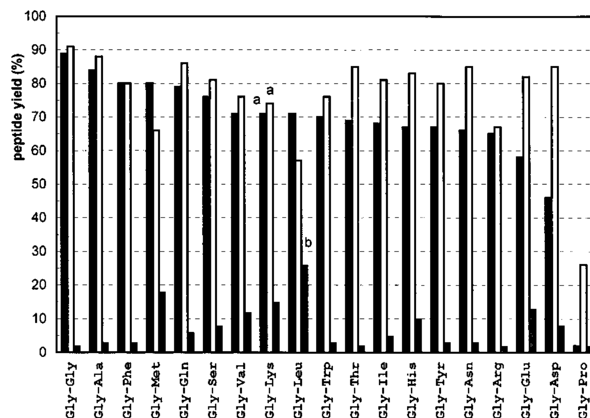

Figure 3 Peptide syntheses catalysed by subtilisin *Carlsberg*, using Z-Val-Trp-OMe as acyl donor, and a series of Gly-Xaa dipeptides as nucleophiles in DMF/0.2 M sodium veronal, pH 9.5 (1/1) ■ and acetonitrile/DMF/H_2O (8/1/1) □; grey bars represent side reactions occurring in the medium of □. Corresponding side reactions in ■ were negligible. (a) Yield represents the sum for the formation of α- and ε-isomers. The predominant constituent of these side products (b) was isolated by preparative HPLC, and its structure was determined by amino acid analysis and FAB-MS to be Z-Val-Trp-Gly-Leu-OMe.

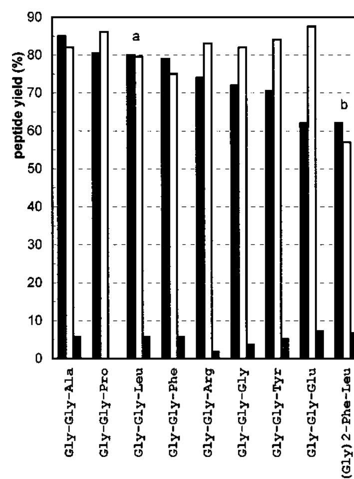

Figure 4 Peptide syntheses catalysed by subtilisin *Carlsberg*, using Z-Val-Trp-OMe as acyl donor, and a series of Gly-Gly-Xaa tripeptides and Gly-Gly-Phe-Leu as nucleophiles in DMF/0.2 M sodium veronal, pH 9.5 (1/1) ■ and acetonitrile/DMF/H_2O (8/1/1) □; grey bars represent side reactions occurring in the medium of □. Corresponding side reactions in ■ were negligible. Z-Val-Trp-Gly-Gly-Leu-OH (a) and Z-Val-Trp-Gly-Gly-Phe-Leu-OH (b) were isolated by preparative HPLC, and their identities were confirmed by amino acid analysis and FAB-MS.

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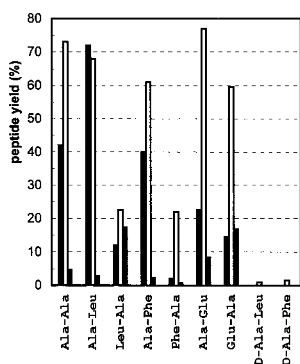

Figure 5 Peptide syntheses catalysed by subtilisin *Carlsberg*, using Z-Val-Trp-OMe as acyl donor, and a series of Ala containing dipeptides as nucleophiles in DMF/0.2 M sodium veronal, pH 9.5 (1/1) ■ and acetonitrile/DMF/H_2O (8/1/1) □; grey bars represent side reactions occurring in the medium of □.

amino acid residues situated in P2′ of the peptide [25]. Such a synergism is efficient, provided that the S1′–P1′ interaction is effective. Controversially, the result of the reactions with Gly-Xaa series catalysed by subtilisin *Carlsberg*, depicted in Figure 3, indicates no stringent specificity for the P2′ residue. The peptides with the hydrophobic bulky amino acid residues in P2′ were not the most efficient nucleophiles of this series. In our case, such synergism between S1′ and S2′ subsites does not play the crucial role in the interaction with the nucleophiles. For example, the nucleophilic behaviour of pairs like Gly-Leu, Leu-Gly and Gly-Phe, Phe-Gly (Figures 2 and 3) or Ala-Leu, Leu-Ala and Ala-Phe, Phe-Ala (Figure 5) was, above all, influenced by the willingness of S1′ to accept only small amino acid residues. The acceptance of dipeptides with charged amino acid residues in the P2′ position was not influenced by electrostatic interaction with S2′, as confirmed by similar yields with other tested dipeptides. The sterically unfavourable Gly-Pro gave only a trace amount of the product. Subtilisin *Carlsberg* exhibited no restriction toward the P3′ position of Gly-Gly-Xaa tripeptides (including Pro residue), resulting in fairly high yields of the products.

Reactions of Z-Val-Trp-OMe with Various Nucleophiles in Low H_2O Medium

The reaction medium consisted of acetonitrile/DMF/H_2O (8/1/1) mixture, in which all educts were soluble. Nucleophiles used in the form of hydrochlorides or peptides with free carboxylic groups were combined with piperidine. In such a strongly basic organic medium, the rate of subtilisin catalysed peptide bond formation was lower by one order of magnitude than in aqueous medium; nevertheless, the enzyme is active within the period necessary to complete the conversion of the acyl component. As a result of the diminished H_2O content, the aminolysis/hydrolysis ratio in the acyl transfer reaction increased in the case of all the nucleophiles tested, as compared with the ratio obtained in an aqueous medium, as demonstrated in Figures 1–5. However, the yields of expected peptide products were negatively influenced by the formation of side products (Figure 3), mainly as a result of enzymatic esterification and oligomerization [26]. In this medium, amino acid amides with bulky hydrophobic or aromatic side chains were the weakest nucleophiles of this series (Figure 1). No product corresponding to the acylation of proline amide was detected. The moderate acylation of Glu(Asp)-NH_2 that was accomplished represents a remarkable exception in enzymatic peptide synthesis. Nucleophiles with a negatively charged amino acid residue in P1′ position are usually not accepted in the acyl transfer reaction catalysed by commonly used proteases [27]. The poor nucleophilic behaviour of Xaa-Gly peptides was improved in organic media only to a limited extent. Nevertheless, the narrow S1′ site of the enzyme was still unable to accommodate bulky [16,21,22] hydrophobic or aromatic amino acid residues. The nucleophilic behaviour of Gly-Xaa peptides was, with the exception of Gly-Pro, almost independent of the structure of the Xaa residue. The S2′ site of the enzyme is a hydrophobic surface along the Asn218-Gly219 backbone of the enzyme flanked by Phe189. It prefers large hydrophobic residues and can accept a variety of others [16,21]. The results of the reactions with dipeptide amides (Table 1) indicate that the rejection of large hydrophobic residues by the S1′ site governs the nucleophilic behaviour of peptides having hydrophobes in both P′ sites. In addition, the syntheses of expected tetrapeptide amides were accompanied by the formation of side products as a result of *C*-terminal peptide deamidation (predominant) and transamidation also leading to the formation of hexapeptides [26]. The hexapeptides and tetrapeptide methyl esters were the main constituents of the side products in the reactions, with some Gly-Xaa dipeptides (Figure 3). The inability to form a hydrogen bond between the imino group of Pro in P2′ and the backbone CO group of Asn218 may be the reason that the Gly-Pro is not well accepted by S′ sites of the enzyme [21,28]. The composition of the

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Table 1 Subtilisin *Carlsberg*-catalysed Reactions of Z-Val-Trp-OMe with Dipeptide Amides in Acetonitrile/DMF/$H_2$0 (8/1/1) (a) and in DMF/0.2 M Sodium Veronal, pH 9.5 (1/1) (b)

Dipeptide amide · HCl		Composition of the reaction mixture (%)				
		Z-2-OH	Z-4-NH_2	Z-4-OH	Z-6-NH_2	Z-6-OH
Gly-Gly-NH_2	a	7	86	5	2	
	b	25	75			
Gly-Leu-NH_2	a	27	20	32.5	28.5	10
	b	31	0.5	42.5	26	
Gly-Phe-NH_2	a	23.5	1	22.5	49.5	3.5
	b	18	1	74.5	6.5	
Ala-Phe-NH_2	a	61.5	3.5	31	2	2
Val-Phe-NH_2	a	94	6			
Phe-Leu-NH_2	a	92	7	1		
Arg-Asn-NH_2	a	76.5	23.5			
	b	95.5	4.5			

Z-2-OH, Z-Val-Trp-OH; Z-4-NH_2, expected product as Z-Val-Trp-Gly-Leu-NH2; Z-4 OH is a result of the product deamidation as Z-Val-Trp-Gly-Leu-OH; Z-6-NH_2 and Z-6-OH are products of oligomerization as Z-Val-Trp-Gly-Leu-Gly-Leu-NH_2 (-OH).

medium had only a minor influence on the yield of peptide products synthesized from the series of Gly-Gly-Xaa nucleophiles (Figure 4). The results of kinetic studies [29] showed that the subtilisin S3′ site was very flexible, and could accommodate different amino acid residues. The results of the reactions between the dipeptides Ala-Leu and Leu-Ala or Ala-Phe and Phe-Ala (Figure 5) confirmed certain synergism between S1′ and S2′ subsites. Nevertheless, S1′ rejected all bulky hydrophobes. Negligible acylation of diastereoisomeric D-Ala-Leu and D-Ala-Phe dipeptides (Figure 5) supported the finding that D-L dipeptides were not acylated by common proteases in acyl transfer reactions [30]. On the other hand, D-amino acid amides could be tolerated by S1′ subsites of proteases [30]. Nevertheless, in our experiments D-Leu-NH_2 proved to be inferior to its L-counterpart as a nucleophile (Figure 1). The acylation of Bu^t esters of amino acids was impossible (not shown). The three-dimensional structure of subtilisin *Carlsberg* in acetonitrile was found to be the same as in H_2O [31]. This would explain the same trend of its nucleophilic specificity in both examined reaction media (Figures 1–5).

Reactions of Z-Ala-Phe-OMe with Amino Acid Derivatives in Various Media

In this set of experiments, we focused on the subtilisin catalysed acylation of bulky hydrophobic phenylalanine amide and proline derivatives. In the case of Phe-NH_2 as an amino component, we tried to improve the yield of the peptide product by searching for a suitable reaction medium. In the case of proline derivatives, we wanted to substantiate our knowledge that subtilisin *Carlsberg* was not able to acylate proline derivatives used in enzymatic peptide synthesis as amino components. Parallel reactions were carried out with Gly-NH_2 as blank experiments. The results (Table 2) showed that peptide synthesis involving the weak nucleophile Phe-NH_2 improved to some extent by controlling the H_2O amount in the organic medium. However, the application of anhydrous organic solvent resulted in a very slow course of the reaction. The yields of peptide syntheses with Phe-NH_2 and other hydrophobic amino acid amides catalysed by modified subtilisin *Carlsberg* in organic solvent [32] or in heterogeneous mixtures [18] were similar. Hydrophobic amino acid amides proved to be very poor nucleophiles in the synthetic reaction catalysed by native subtilisin *Carlsberg* [14]. On the other hand, high synthetic yields with hydrophobic nucleophiles using subtilisin BPN′ and other enzyme variants [17,18,33,34] were reported. Our attempts to achieve peptide bond formation catalysed by subtilisin *Carlsberg* using Pro-NH_2 or Pro-OBzl as amino components failed, regardless of the conditions used (Table 2). We did not detect any of the expected peptide products by HPLC. The description of subtilisin *Carlsberg* catalysed peptide synthesis of Z-Ala-Phe-Pro-NH_2, starting from Z-Ala-Phe-OMe and Pro-NH_2 in anhydrous Bu^t-OH was very

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Table 2 Subtilisin *Carlsberg*-catalysed Tripeptide Synthesis Using Z-Ala-Phe-OMe as Acyl Donor and Gly-NH_2, Phe-NH_2, Pro-NH_2 and Pro-OBzl as Nucleophiles in Various Media

Reaction medium	Nucleophile (X)	Yield (%) Z-Ala-Phe-X/Z-Ala-Phe-OH			
		1 h	24 h	48 h	72 h
DMF/0.2 M Na-veronal, pH 9.5 (1/1)	H-Gly-NH_2	87/13	–	–	–
	H-Phe-NH_2	3/97	–	–	–
	H-Pro-NH_2	0/100	–	–	–
Acetonitrile/H_2O (9/1)	H-Gly-NH_2	98/2	–	–	–
	H-Phe-NH_2	7/93	–	–	–
	H-Pro-NH_2	0/100	–	–	–
	H-Pro-OBzl	0/99	–	–	–
Acetonitrile/H_2O (49/1)	H-Gly-NH_2	99/1	–	–	–
	H-Phe-NH_2	48/52	56/44	–	–
	H-Pro-NH_2	0/97	0/99	–	–
	H-Pro-OBzl	0/5	0/66	–	–
Bu^t-OH/H_2O (49/1)	H-Gly-NH_2	95/2	96/3	–	–
	H-Phe-NH_2	25/25	67/33	–	–
	H-Pro-NH_2	0/77	0/100	–	–
	H-Pro-OBzl	0/1	0/99	–	–
Bu^t-OH	H-Gly-NH_2	3/0	35/0	68/1	94/2
	H-Phe-NH_2	1/1	12/5	22/8	31/11
	H-Pro-NH_2	0/0	0/24	0/44	0/53
	H-Pro-OBzl	0/0	0/9	0/14	0/18

convincing [19]. According to Chen's paper [19], the tripeptide was obtained in 86% preparative yield. We reproduced his experiment precisely. However, no product was isolated using the same work up procedure. Moreover, the competitive hydrolysis of Z-Ala-Phe-OMe, as determined by HPLC, was very slow. The description of another subtilisin catalysed synthesis using Pro-OBzl was somewhat dubious [19]. We, therefore, slightly modified the experiment, trying to synthesize Z-Ala-Phe-Pro-OBzl in Bu^t-OH containing 2% of H_2O. No HPLC-peak of tripeptide was found, apart from the peaks of Z-Ala-Phe-OMe(-OH). The data published about the specificity of subtilisins stated that no hydrolysis of -Xaa-Pro- peptide bond of synthetic peptides as a substrate [28,35,36] was observed. These facts support our conclusion that the enzyme is not capable of catalysing the synthesis of the -Xaa-Pro- peptide bond.

Comparison of Nucleophile Specificity of Subtilisin *Carlsberg* and Subtilisin BPN′ in Enzymatic Peptide Synthesis

We had studied the nucleophile specificity of subtilisin BPN′ in an organic solvent with low H_2O content via acyl transfer reaction, using Ac-Tyr-OEt as an acyl donor [12]. Values of partition constants determined for identical nucleophiles show a similar trend of nucleophile specificity [12], as obtained for the yield values in the present study. The primary structure of the *Carlsberg* enzyme differs from the BPN′ enzyme, but the tertiary structures of both enzymes are very similar. The replacement of the relatively bulky Tyr 217 at the end of S1′ region of BPN′ by the smaller Leu residue in *Carlsberg* should increase the space for the accommodation of bulky residues to a certain degree [22]. We speculated that such a minute difference in the S1′ site should not influence the nucleophile specificity between these species. The yields of the of Xaa-Gly (Xaa: Leu, Ile, Phe, Tyr, Trp) acylation was very low for all dipeptides in the case of the *Carlsberg* enzyme (Figure 2). Although values of partition constants of these five dipeptides determined in the acyl transfer reaction catalysed by subtilisin BPN′ exhibited remarkable differences [12], all five were poor nucleophiles. The success of some subtilisin catalysed syntheses using bulky hydrophobic nucleophiles was, above all, influenced by the reaction conditions. However, we can not explain remarkable differences in the nucleophilic behaviour of negatively

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charged Asp(G1u)-Gly dipeptides between the BPN′ [12] and *Carlsberg* enzyme.

Enzymatic Hydrolysis of *N*-terminal Nonapeptide Methyl Ester of Eel Calcitonin

The *C*-terminal deprotection of cyclo[1-6]-Ser(Bu^t)-Asn-Leu-Ser(Bu^t)-Thr(Bu^t)-Asu-Val-Leu-OMe was done under mild alkaline conditions, with the help of subtilisin in aqueous methanol. Under the experimental conditions used, the ester was totally hydrolysed, and the internal peptide bonds were not affected by the enzyme. We avoided Asn deamidation, which would result from usual alkaline saponification. The hydrolysed nonapeptide was used for the chemical coupling with the *C*-terminal segment of the calcitonin molecule, which will be described in another paper. In a comparative experiment, the enzymatic hydrolysis of the side chain deprotected cyclo[1-6]-Ser-Asn-Leu-Ser-Thr-Asu-Val-Leu-OMe resulted in additional cleavage of the Leu-Ser peptide bond. This gives evidence of the enzyme's preference for small hydrophilic residues in P1′. The protection of the Ser side chain by a bulky group hinders enzymatic cleavage at this point.

CONCLUSIONS

The nucleophilic specificity of subtilisin *Carlsberg* represents a crucial point in its application in enzymatic peptide synthesis. The enzyme accepts numerous peptides and amino acid derivatives as nucleophiles with a strong preference for the Gly residue in the P1′ position. Amino acids with hydrophilic and charged side chains in this position are accepted better than those with bulky hydrophobic side chains. Contrary to the published data [19], subtilisin is not capable of synthesizing the -Xaa-Pro- peptide bond.

The enzyme is substantially more flexible with respect to different amino acid residues in the P2′ position, with the exception of Pro. No restrictions were observed toward the P3′ position. The yields of peptide synthesis can be substantially enhanced by employing organic media with restricted H_2O content. However, side reactions, such as the acylation of the lysine ε-amino group, splitting of the *C*-terminal amide, transamidation, formation of oligomeric products, esterification of the *C*-terminal carboxylic group and non-specific hydrolysis of internal peptide bonds represent another obstacle for the application of the enzyme, particularly in segment condensations. Broad substrate specificity, together with high enzymatic stability in organic solvents, makes the enzyme an extremely useful tool for the deprotection of *C*-terminal esters of protected peptides in solution phase peptide chemistry. We do not see remarkable differences in nucleophile specificity between the *Carlsberg* and BPN′ enzymes, nor in the possibilities of their application in peptide synthesis.

Acknowledgements

This work was supported by the grant agency of the Czech Republic (grant 203/95/0014). We thank Dr. Linda Hauserova, for reading and correcting the manuscript.

REFERENCES

1. Bongers J, Heimer EP. Recent applications of enzymatic peptide synthesis. *Peptides* 1994; **15**: 183–193.
2. Schellenberger V, Jakubke H-D. Protease-catalyzed kinetically controlled peptide synthesis. *Angew. Chem. Int. Ed. Eng.* 1991; **30**: 1437–1449.
3. Čeřovský V, Martinek K. Free chymotrypsin-catalyzed synthesis of peptide bond in aliphatic alcohols with low water content. *Collect. Czech. Chem. Commun.* 1989; **54**: 266–276.
4. Clapés P, Adlercreutz P, Mattiasson B. Enzymatic peptide synthesis in organic media: a comparative study of water-miscible and water-immiscible solvent systems. *J. Biotechnol.* 1990; **15**: 323–338.
5. Clapès P, Torres J-L, Adlercreutz P. Enzymatic peptide synthesis in low water content systems: preparative enzymatic synthesis of [Leu]- and [Met]-enkephalin derivatives. *Bioorg. Med. Chem.* 1995; **3**: 245–255.
6. Margolin AL, Tai D-F, Klibanov AM. Incorporation of D-amino acids into peptides via enzymatic condensation in organic solvents. *J. Am. Chem. Soc.* 1987; **109**: 7885–7887.
7. Barbas CF, Matos JR, West JB, Wong CH. A search for peptide ligase: cosolvent-mediated conversion of proteases to esterases for irreversible synthesis of peptides. *J. Am. Chem. Soc.* 1988; **110**: 5162–5166.
8. Čeřovský V, Jakubke H-D. Enzymatic approach to the synthesis of taurine-containing peptides. *Int. J. Peptide Protein Res.* 1994; **44**: 466–471.
9. Markland FS, Smith EC. Subtilisins: primary structure, chemical and physical properties. In *The Enzymes*, vol. 3, Boyer PD (ed). Academic Press: New York, 1971; 561–608.

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10. Ottesen M, Svendsen I. The subtilisins. In *Methods in Enzymology*, vol. 19, Perlmann GE, Lorand L (eds). Academic Press: New York, 1970; 199–215.
11. Russell AJ, Fersht AR. Commercial samples of subtilisin BPN′. *Nature* 1986; **321**: 733.
12. Čeřovský V, Jakubke H-D. Nucleophile specificity of subtilisin in an organic solvent with low water content: investigation via acyl transfer reactions. *Biotechnol. Bioeng.* 1996; **49**: 553–558.
13. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* 1967; **27**: 157–162.
14. Kise H, Fujimoto K. Enzymatic reactions in aqueous-organic media VIII. Medium ellect and nucleophile specificity in subtilisin-catalyzed peptide synthesis in organic solvents. *Biotechnol. Lett.* 1988; **10**: 883–888.
15. Gololobov MY, Voyushina TL, Stepanov VM, Adlercreutz P. Nucleophile specificity in α-chymotrypsin- and subtilisin- (*Bacillus subtilis* strain 72) catalyzed reactions. *Biochim. Biophys. Acta.* 1992; **1160**: 188–192.
16. Moree WJ, Sears P, Kawashiro K, Witte K, Wong C-R. Exploration of subtilisin BPN′ as catalyst for the synthesis of peptides containing non-coded amino acids, peptide mimetics and peptide conjugates. *J. Am. Chem. Soc.* 1997; **119**: 3942–3947.
17. Wang P, Hil TG, Wartchow CA, Huston ME, Oehler LM, Smith MB, Bednarski MD, Callstrom MR. New carbohydrate-based materials for the stabilization of proteins. *J. Am. Chem. Soc.* 1992; **114**: 378–380.
18. Gill I, Vulflon EN. Enzymatic synthesis of short peptides in heterogeneous mixtures of substrates. *J. Am. Chem. Soc.* 1993; **115**: 3348–3349.
19. Chen S-T, Kao C-L, Wang K-T. Alkaline protease catalysis of a secondary amine to form a peptide bond. *Int. J. Peptide Protein Res.* 1995; **46**: 314–319.
20. Čeřovský V, Wünsch E, Brass J. Enzymatic semisynthesis of dicarba analogs of calcitonin. *Eur. J. Biochem.* 1997; **247**: 231–237.
21. Robertus JD, Kraut J, Alden RA, Birktoft JJ. Subtilisin; a stereochemical mechanism involving transition-state stabilization. *Biochemistry* 1972; **11**: 4293–4303.
22. Bonneau PR, Graycar TP, Estell DA, Jones LB. Alteration of the specificity of subtilisin BPN′ by site-directed mutagenesis in its S1 and S1′ binding sites. *J. Am. Chem. Soc.* 1991; **113**: 1026–1030.
23. Kitaguchi H, Ono M, Itoh I, Klibanov AM. Regioselective modification of lysine's amino groups by proteases. *Agric. Biol. Chem.* 1991; **55**: 3067–3073.
24. Čeřovský V, Jakubke H-D. Acyl transfer reactions catalyzed by native and modified α-chymotrypsm in acetonitnle with low water content. *Enzyme Microb. Technol.* 1994; **16**: 596–601.
25. Bratovanova EK, Petkov DD. Glycine flanked by hydrophobic bulky amino acid residues as minimal sequence for effective subtilisin catalysis. *Biochem. J.* 1987; **248**: 957–960.
26. Čeřovský V, Martinek K. Peptide synthesis catalyzed by native proteinase K in water-miscible organic solvents with low water content. *Collect. Czech. Chem. Commun.* 1989; **54**: 2027–2041.
27. Capellas M, Caminal G, Gonzales G, Lopez-Santin J, Clapés P. Enzymatic condensation of cholecystokinin CCK-8 (4-6) and CCK-8 (7-8) peptide fragments in organic media. *Biotechnol. Bioeng.* 1997; **56**: 456–463.
28. Morihara K, Oka T. A kinetic investigation of subsites S1′ and S2′ in α-chymotrypsin and subtilisin BPN′. *Arch. Biochem Biophys.* 1977; **178**: 188–194.
29. Grøn H, Meldal M, Breddam K. Extensive comparison of the substrate preferences of two subtilisins as determined with peptide substrates which are based on the principle of intramolecular quenching. *Biochemistry* 1992; **31**: 6011–6018.
30. Čeřovský V. Serine proteinase-catalyzed incorporation of D-amino acids into model peptides in acetonitrile with low water content. *Biomed. Biochim. Acta* 1991; **50**: 44–49.
31. Fitzpatrick PA, Steinmetz ACU, Ringe D, Klibanov AM. Enzyme crystal structure in a neat organic solvent. *Proc. Nad. Acad. Sci. USA* 1993; **90**: 8653–8657.
32. Ferjancic A, Puigserver A, Gaertner H. Subtilisin-catalysed peptide synthesis and transesterification in organic solvents. *Appl. Microbiol. Biotechnol.* 1990; **32**: 651–657.
33. Wong C-H, Chen ST, Hennen WJ, Bibbs JA, Wang Y-F, Liu JL-C, Pantoliano MW, Whitlow M, Bryan PN. Enzymes in organic synthesis: use of subtilisin and a highly stable mutant derived from multiple site-specific mutations. *J. Am. Chem. Soc.* 1990; **112**: 945–953.
34. Getun IV, Filippova IY, Lysogorskaya EN, Oksenoit ES, Anisimova W, Kolobanova SV, Bacheva AV, Stepanov VM. SDS-subtilisin complex efficiently catalyzes synthesis of peptides in ethanol and 2-propanol. *Bioorg. Med. Chem. Lett.* 1997; **7**: 2691–2696.
35. Brömme D, Peters K, Fink S, Fittkau S. Enzyme–substrate interactions in the hydrolysis of peptide substrates by thermitase, subtilism BPN′ and proteinase K. *Arch. Biochem. Biophys.* 1986; **244**: 439–446.
36. Abrahmsén L, Tom J, Burnier J, Butcher KA, Kossiakoff A, Wells JA. Engineering subtilisin and its substrates for efficient ligation of peptide bonds in aqueous solution. *Biochemistry* 1991; **30**: 4151–4159.

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