Investigations on the Enzyme Specificity of Clostripain: A New Efficient Biocatalyst for the Synthesis of Peptide Isosteres†

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To explore the ability of the cysteine protease clostripain as a biocatalyst for the synthesis of peptide isosteres, the S′-subsite specificity of this enzyme toward unnatural substrates was investigated. First, the function of clostripain for acylating aliphatic noncyclic and cyclic amines varying in chain length and ring size was analyzed using a standard acyl donor. Additionally, this series was expanded by use of aromatic amines, amino alcohols, derivatives of non-α-amino carboxylic acids, and symmetric and asymmetric diamines, respectively. The results obtained give a detailed picture of the unique reactivity of clostripain toward synthetic substrates, allowing insights into the basic enzyme−substrate interactions. Furthermore, the data provide a guideline for the use of clostripain as a biocatalyst for synthesis of peptide isosteres. The study was completed by the utilization of a model substrate mimetic enabling clostripain to react with noncoded and non-amino acid-derived amines as well as nonspecific acyl moieties. The results of this study indicate that this approach may extend the application range of clostripain as a biocatalyst outside of peptide synthesis.

Introduction

It is well-documented that proteases catalyze in vitro the hydrolysis of peptides and proteins as well as the reverse of hydrolysis.[1] On the basis of this activity, these enzymes can be used as biocatalysts for peptide bond formations apparently opposite to their original in vivo function. Due to the mild synthesis conditions and the high degree of stereo- and regiospecificity of such reactions, enzymatic peptide coupling is considered to be an attractive alternative for solution and solid phase peptide synthesis. However, despite these undisputed advantages, the high substrate specificity of proteases seriously limits the amino acid residues between which a peptide bond can be synthesized. Due to the original specificity of proteases for proteinogenic amino acids, the coupling of modified and noncoded amino acids or non-amino acid-derived derivatives is especially difficult. Therefore, only a few papers report the protease-mediated synthesis of peptides containing artificial amino acids or non-amino acid-derived functionalities.[2] But even in these cases the product yields usually drop with an increasing degree of modifications of both the amino acid side chain and the backbone structure particularly in the region of synthesis.[3]

Peptide isosteres have become important for the synthesis of pharmacologically active and proteolytically stable peptide derivatives[4] or protease inhibitors.[5] Hence, there is a strong need for efficient, selective, and environmentally friendly methods for their synthesis. Thus, the screening of naturally occurring proteases is fundamental to find suitable biocatalysts possessing appropriate catalytic properties. Our results found for clostripain-catalyzed couplings of trifluoromethyl-substituted peptides[2g] indicate a high reactivity of this cysteine protease toward synthetically modified amino acid-containing acyl acceptors. Motivated by these findings, the S′-subsite specificity (nomenclature according to ref 6) of clostripain was studied in detail by acyl transfer experiments to libraries of unnatural amino components

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† Abbreviations: Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; DTT, DL-dithiothreitol; HEPES, (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]); OEt, ethyl ester; OGp, 4-guanidinophenyl ester; Z, benzyloxycarbonyl.

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(1) (a) Schellenberger, V.; Jakubke, H.-D. *Angew. Chem.* **1991**, *103*, 1440; *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1437. (b) Wong, C. H. *Science* **1989**, *244*, 1145. (c) Jackson, D. Y.; Burnier, J.; Quan, C.; Stanley, M.; Tom, J.; Wells, J. A. *Science* **1994**, *266*, 243. (d) Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon: Oxford, 1994; pp 41−130 and references therein.

(2) (a) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1981**, *46*, 97. (b) Cerovsky, V.; Jakubke, H.-D. *Int. J. Pept. Protein Res.* **1994**, *44*, 466. (c) Margolin, A. L.; Tai, D.-F.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 7885. (d) Schuster, M.; Munoz, B.; Yuan, W.; Wong, C.-H. *Tetrahedron Lett.* **1993**, *34*, 1247. (e) Wong, C.-H.; Schuster, M.; Wang, P.; Sears, P. *J. Am. Chem. Soc.* **1993**, *115*, 5893. (f) Schuster, M.; Wang, P.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 1135. (g) Bordusa, F.; Dahl, C.; Jakubke, H.-D.; Burger, K.; Koksch, B. *Tetrahedron: Asymmetry* **1999**, *10*, 307.

(3) (a) Chen, S. T.; Chen, S. Y.; Wang, K. T. *J. Org. Chem.* **1992**, *57*, 6960. (b) Moree, W. J.; Sears, P.; Kawashiro, K.; Witte, K.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 3942. (c) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272. (d) Cantacuzene, D.; Guerreiro, C. *Tetrahedron* **1989**, *45*, 741.

(4) (a) Jakubke, H.-D. *Peptide, Chemie und Biologie*; Spektrum Akademischer Verlag: Heidelberg, Berlin, Oxford, 1996. (b) Valle, G.; Crisma, M.; Toniolo, C.; Polinelli, S.; Boesten, W. H. J.; Schoemaker, H. E.; Meijer, E. M.; Kamphius, J. *Int. J. Peptide Protein Res.* **1991**, *37*, 521. (c) Benedetti, E. *Biopolymers* **1996**, *40*, 3. (d) Karle, I. L. *Biopolymers* **1996**, *40*, 157. (e) Giannis, A.; Kolter, T. *Angew. Chem.* **1993**, *105*, 1303; *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1244.

(5) (a) Rich, D. H. In *Comprehensive Medicinal Chemistry*; Sammes, P. G., Ed.; Pergamon: Oxford, 1990; Vol. 2, p 391. (b) Alewood, P. F.; Brinkworth, R. I.; Dancer, R. J.; Garnham, B.; Jones, A.; Kent, S. B. H. *Tetrahedron Lett.* **1992**, *33*, 977. (c) Scholz, D.; Billich, A.; Charpiot, B.; Ettmayer, P.; Lehr, P.; Rosenwirth, B.; Schreiner, E.; Gstach, H. *J. Med. Chem.* **1994**, *37*, 3079. (d) Slee, D. H.; Laslo, K. L.; Elder, J. H.; Ollmann, I. R.; Gustchina, A.; Kervinen, J.; Zdanov, A.; Wlodawer, A.; Wong, C.-H. *J. Am. Chem. Soc.* **1995**, *117*, 11867.

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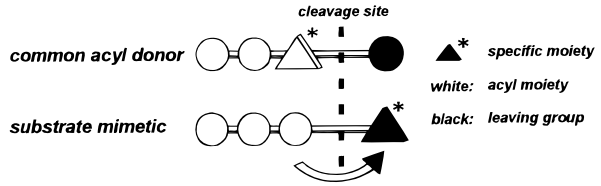
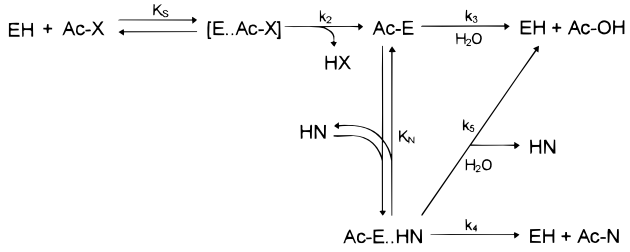


Figure 1. Schematic structure of substrate mimetics compared to common acyl donor components.

Scheme 1. Kinetic Model of Protease-Catalyzed Acyl Transfer Reaction[a]



[a] EH, free enzyme; Ac-X, acyl donor; HX, leaving group; Ac-E, acyl enzyme complex; Ac−OH, hydrolysis product; HN, acyl acceptor; Ac−N, aminolysis product.

derived from noncoded amino acids, cyclic, noncyclic, and aromatic amines, amino alcohols, and symmetric and asymmetric diamines, respectively. Clostripain, produced and secreted by proliferating cells of the anaerobic microorganism *Clostridium histolyticum*, originally hydrolyzes peptide bonds at the carboxyl side of arginine and, much more slowly, of lysine residues.[7] For that reason, we started our study using the conventional substrate Bz-Arg-OEt as the acyl donor and expanded it to the substrate mimetic Bz-Phe-OGp (Figure 1).[8] The latter represents a new type of acyl donors which are recognized by the enzyme on the basis of specific interactions with the 4-guanidinophenyl ester leaving group (OGp). This highly specific leaving group binds instead of the specificity-determining arginine side chain functionality of common acyl donors largely independently of its C-terminal amino acid residue. As a result, peptide bond formation occurs independently of the primary specificity of the enzyme, enabling clostripain to react also with nonspecific acyl moieties.[9]

Results and Discussion

Deacylation Kinetics. In the presence of added nucleophilic amino components, cysteine and serine proteases are capable of catalyzing acyl transfers to nucleophiles simultaneously with the hydrolysis of the acyl donors (Scheme 1). Consequently, the resulting hydrolysis product Ac−OH, which possesses a very low acylation potential, and the resulting peptide product Ac−N are formed. As an efficiency parameter of the two competitive reactions, the partition value *p* was introduced analogously to the definition of the Michaelis constant K_M (eq 1).[10]

$$p = \frac{d[\mathrm{Ac{-}OH}]}{d[\mathrm{Ac{-}N}]}[\mathrm{HN}] = \frac{k_3 K_n}{k_4} + \frac{k_5}{k_4}[\mathrm{HN}] = p_0 + p_n[\mathrm{HN}] \quad (1)$$

According to eq 1, the *p* value is defined as the nucleophile concentration at which the rates of both reactions are equal corresponding to 50% yield. Consequently, a decrease in the *p* value is inversely correlated with an increase in the peptide yield. Using the nucleophile in excess, the partition value can be determined from the product ratios according to eq 2, where [HN] is

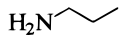
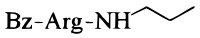
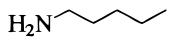
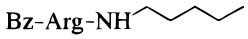
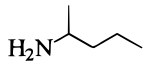
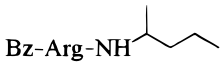
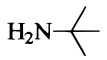
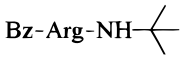
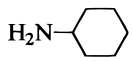
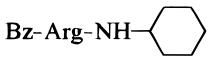
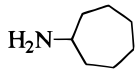
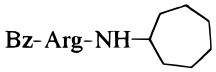
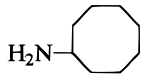
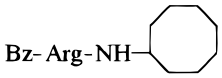
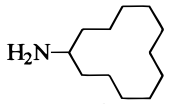
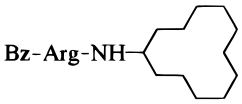
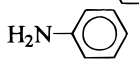
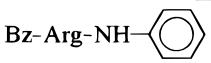
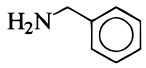
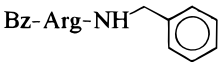
$$p = \frac{[\mathrm{Ac{-}OH}]}{[\mathrm{Ac{-}N}]}[\mathrm{HN}] \quad (2)$$

the initial nucleophile concentration and [Ac−OH] and [Ac−N] represent the product concentrations.

Acyl Transfer Studies. The ability of clostripain to catalyze acylations of a wide variety of nucleophilic components was explored using Bz-Arg-OEt as the acyl donor and about 40 amines of different structure as acyl acceptors. At first, the function of clostripain for coupling of aliphatic noncyclic and cyclic amines varying in chain length or ring size was investigated. Additionally, this series was expanded by the use of two aromatic amines (compounds **9** and **10**). The resulting partition values of coupling reactions are summarized in Table 1. The data document that, except for compound **4**, all amines show productive binding at the S′-subsite of clostripain resulting in amide product formation. Remarkably, *p* values within a range of approximately 1 to 5 mM were evident for most derivatives corresponding to results found for most efficient peptidic acyl acceptors.[7] This fact also holds for the acylation of the bulky cyclic amines **5**−**7** at least to a ring size of eight methylene groups. Since these substrates do not have a common peptide structure, this finding suggests that clostripain does not form close contacts to the backbone, in particular to the amide function of P'_2 amino acid residues, contrary to most other proteases.[11] Instead of this, the high reactivity toward these bulky hydrophobic amines indicates a more flexible and relaxed S′-subsite binding region. Only the use of cyclododecylamine (compound **8)** drops the efficiency of catalysis indicated by the increase in *p*. In this case mainly competitive hydrolysis of the acyl donor was observed. An increase of the *p* value was also found for the branched noncyclic 2-aminopentane (**3**). In contrast, the nonbranched analogues **1** and **2** gave more than 1 order of magnitude lower partition values, indicating an unfavorable effect of a neighboring branch on the CN-bond formation. Further alkylation of the neighboring carbon resulting in dialkylated amines causes a complete loss of reactivity as found for reactions with *tert*-butylamine (**4**) as the acyl acceptor. This observation corresponds with the failed acceptance of clostripain for the $C^{\alpha,\alpha}$-dialkylated amino acid analogue H-Aib-NH_2.[2g] The comparatively high *p* value found for aniline (**9**), which is similar to that of 2-aminopentane (**3**), might also be

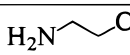
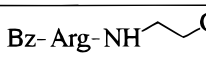
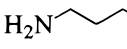
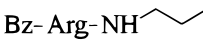
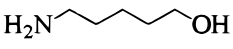
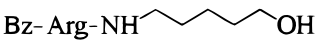
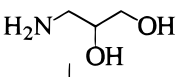
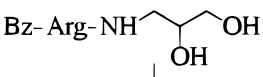
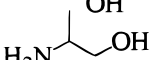
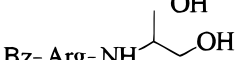
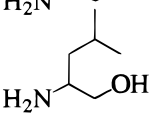
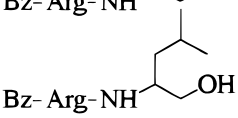
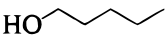
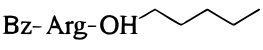
(6) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
(7) (a) Ullmann, D.; Jakubke, H.-D. *Eur. J. Biochem.* **1994**, *223*, 865. (b) Bordusa, F.; Ullmann, D.; Jakubke, H.-D. *Biol. Chem.* **1997**, *378*, 193.
(8) Thormann, M.; Thust, S.; Hofmann, H.-J.; Bordusa, F. *Biochemistry* **1999**, *38*, 6056.
(9) Bordusa, F.; Ullmann, D.; Elsner, C.; Jakubke, H.-D. *Angew. Chem.* **1997**, *109*, 2583; *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2473.
(10) Könnecke, A.; Schellenberger; V.; Hofmann, H.-J.; Jakubke, H.-D. *Pharmazie* **1984**, *39*, 785.
(11) (a) Segal, D. M.; Powers, J. C.; Cohen, G. H.; Davies, D. R.; Wilcrox, P. E. *Biochemistry* **1971**, *10*, 3728. (b) Bizzozero, S. A.; Baumann, W. K.; Dutler, H. *Eur. J. Biochem.* **1982**, *122*, 251.

Table 1. Partition Values *p* of Clostripain-Catalyzed Acyl Transfer Reactions of Bz-Arg-OEt with Alkyl and Aryl Amines[a]

acyl donor	acyl acceptor		product		*p* value [mM]
Bz-Arg-OEt		(1)		(11)	0.80
Bz-Arg-OEt		(2)		(12)	1.51
Bz-Arg-OEt		(3)		(13)	17.0
Bz-Arg-OEt		(4)		(14)	no synthesis
Bz-Arg-OEt		(5)		(15)	3.32
Bz-Arg-OEt		(6)		(16)	2.53
Bz-Arg-OEt		(7)		(17)	2.04
Bz-Arg-OEt		(8)		(18)	126
Bz-Arg-OEt		(9)		(19)	30.2
Bz-Arg-OEt		(10)		(20)	4.61

[a] Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, $[\text{acyl acceptor}]_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

Table 2. Partition Values *p* of Clostripain-Catalyzed Acyl Transfer Reactions of Bz-Arg-OEt with Various Amino Alcohols[a]

acyl donor	acyl acceptor		product		*p* value [mM]
Bz-Arg-OEt		(21)		(28)	2.21
Bz-Arg-OEt		(22)		(29)	3.92
Bz-Arg-OEt		(23)		(30)	2.15
Bz-Arg-OEt		(24)		(31)	2.70
Bz-Arg-OEt		(25)		(32)	9.74
Bz-Arg-OEt		(26)		(33)	1.60
Bz-Arg-OEt		(27)		(34)	no synthesis

[a] Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, $[\text{acyl acceptor}]_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

the result of this unfavorable branch-effect. Correspondingly, in the case of benzylamine (**10**), the shift of the branch by the additional methylene group leads to a significant increase in the degree of amine acylation. Interestingly, a branch with longer aliphatic residues seems to compensate partly for this unfavorable branch-effect, as suggested by the high reactivity of leucinol (**26**) documented in Table 2. Furthermore, Table 2 illustrates the influence of additional functionalities, e.g., hydroxyl groups, within the amino component on clostripain-catalyzed amide formations. Remarkably, in no case a competitive formation of an O-acylated product occurred,

Table 3. Partition Values *p* of Clostripain-Catalyzed Acyl Transfer Reactions of Bz-Arg-OEt with Symmetric and Asymmetric Alkyl Diamines[a]

acyl donor	acyl acceptor		product		*p* value [mM]
Bz-Arg-OEt	H_2N⌒NH_2	**(35)**	Bz-Arg-NH⌒NH_2	**(42)**	0.87
Bz-Arg-OEt	H_2N⌒⌒NH_2	**(36)**	Bz-Arg-NH⌒⌒NH_2	**(43)**	1.09
Bz-Arg-OEt	H_2N⌒⌒NH_2	**(37)**	Bz-Arg-NH⌒⌒NH_2	**(44)**	1.43
Bz-Arg-OEt	H_2N⌒⌒⌒NH_2	**(38)**	Bz-Arg-NH⌒⌒⌒NH_2	**(45)**	9.40
Bz-Arg-OEt	H_2N⌒⌒⌒NH_2	**(39)**	Bz-Arg-NH⌒⌒⌒NH_2	**(46)**	2.11
Bz-Arg-OEt	H_2N⌒⌒⌒⌒NH_2	**(40)**	Bz-Arg-NH⌒⌒⌒⌒NH_2	**(47)**	2.36
Bz-Arg-OEt	H_2N⌒(NH_2)	**(41)**	Bz-Arg-NH⌒(NH_2)	**(48)**	3.56

[a] Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, [acyl acceptor]$_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

as also indicated by the failed acceptance of the enzyme for 1-pentanol (**27**). A comparison of the partition values found for the individual amino alcohols with those observed for the appropriate unsubstituted amines does not show significant differences (cf. Table 1). Hence, clostripain-catalyzed reactions seem to be nearly unaffected by additional hydroxyl groups of the acyl acceptors. This behavior also holds for the acylation of the dihydroxylated 3-amino-1,2-propanediol (**24**). Corresponding to this, reactions using alaninol (**25**) result in *p* values comparable to that found for the non-hydroxylated analogue 2-aminopentane (**3**). Thus, clostripain catalyzes the acylation of amino alcohols not only very efficiently like that of unsubstituted amines, but also with a high selectivity to the amino group of these acyl acceptors.

The selectivity of clostripain catalysis was further investigated by using various aliphatic diamines (**35**–**41**) as acyl acceptors. The results observed for reactions with Bz-Arg-OEt are summarized in Table 3. In most cases, the *p* values are comparable with those of the similar single amines. Obviously, as already found for the extra hydroxyl groups of amino alcohols, further amino groups also appear to have no significant influence on the efficiency of enzyme catalysis, thus qualifying diamines as specific acyl acceptors for clostripain, too. Only the reactions with 1,6-diaminohexane (**38**) resulted in a slightly higher *p* value, indicating unfavorable interactions of the extra amino group with the S′-subsite of clostripain. A partly positive surface charge at the appropriate enzyme region could explain this phenomenon. However, such a positively charged domain should be strong locally restricted, as shown by the results observed for the ω-amino carboxylic acids (Table 4). As indicated by the higher *p* values found for these amino components, a negative surface charge should predominate within the S′-subsite of clostripain. As illustrated in Figure 2, the strongest negative potential should exist very near the active site. Therefore, the shortest substrate is also the poorest one, whereas a shift of the negatively charged carboxylate group to more remote

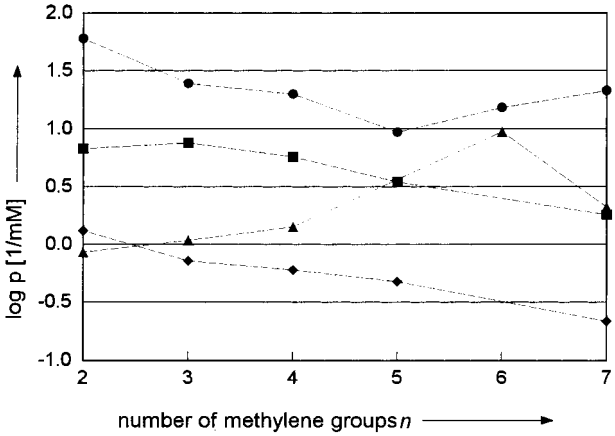


Figure 2. Influence of various functional groups at different positions of the acyl acceptor of the general structure H_2N-$(CH_2)_n$R on log *p* of clostripain-catalyzed acyl transfer reactions. R: (◆) $COOCH_3$; (●) COOH; (■) $CONH_2$; (▲) NH_2. To facilitate the understanding of these semilogarithmic plots, the symbols are connected by dotted lines. Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor]: Bz-Arg-OEt = 2 mM, [acyl acceptor]$_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

positions increases the rate of acylation. The optimum of reactivity found for 6-aminohexanoic acid (**52**) inversely correlates with the lower reactivity observed for 1,6-diaminohexane (**38**), confirming the postulated change of surface charges in the appropriate S′-subsite region. The reactivity of the corresponding carboxylic acid amides (**54**–**58**) and esters (**59**–**63**) are practically unaffected by these electrostatic effects. In both cases the reactivity increases with the number of methylene groups and, therefore, with the degree of hydrophobicity, whereas the esters act as the most efficient substrates. Hence, from a synthetic point of view, the enzymatic acylation of amino carboxylic acids should be performed using the appropriate ester analogues which can be subsequently hydrolyzed to the corresponding free acids.

Table 4. Partition Values *p* of Clostripain-Catalyzed Acyl Transfer Reactions of Bz-Arg-OEt with Non-α-amino Carboxylic Acid Derivatives[a]

acyl donor	acyl acceptor		product		*p* [mM]
Bz-Arg-OEt	H_2N–COOH	**(49)**	Bz-Arg-NH–COOH	**(64)**	62.8
Bz-Arg-OEt	H_2N–COOH	**(50)**	Bz-Arg-NH–COOH	**(65)**	24.4
Bz-Arg-OEt	H_2N–COOH	**(51)**	Bz-Arg-NH–COOH	**(66)**	20.0
Bz-Arg-OEt	H_2N–COOH	**(52)**	Bz-Arg-NH–COOH	**(67)**	9.30
Bz-Arg-OEt	H_2N–COOH	**(53)**	Bz-Arg-NH–COOH	**(68)**	21.2
Bz-Arg-OEt	H_2N–$CONH_2$	**(54)**	Bz-Arg-NH–$CONH_2$	**(69)**	6.73
Bz-Arg-OEt	H_2N–$CONH_2$	**(55)**	Bz-Arg-NH–$CONH_2$	**(70)**	7.62
Bz-Arg-OEt	H_2N–$CONH_2$	**(56)**	Bz-Arg-NH–$CONH_2$	**(71)**	5.74
Bz-Arg-OEt	H_2N–$CONH_2$	**(57)**	Bz-Arg-NH–$CONH_2$	**(72)**	3.50
Bz-Arg-OEt	H_2N–$CONH_2$	**(58)**	Bz-Arg-NH–$CONH_2$	**(73)**	1.83
Bz-Arg-OEt	H_2N–$COOCH_3$	**(59)**	Bz-Arg-NH–$COOCH_3$	**(74)**	1.32
Bz-Arg-OEt	H_2N–$COOCH_3$	**(60)**	Bz-Arg-NH–$COOCH_3$	**(75)**	0.73
Bz-Arg-OEt	H_2N–$COOCH_3$	**(61)**	Bz-Arg-NH–$COOCH_3$	**(76)**	0.61
Bz-Arg-OEt	H_2N–$COOCH_3$	**(62)**	Bz-Arg-NH–$COOCH_3$	**(77)**	0.48
Bz-Arg-OEt	H_2N–$COOCH_3$	**(63)**	Bz-Arg-NH–$COOCH_3$	**(78)**	0.22

[a] Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, [acyl acceptor]$_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

Analyzing the selectivity of enzymatic acylation of the diamines, a single acylation was found in almost all cases. Traces of double-acylated product could only be detected for ethylendiamine (**35**) with approximately 3% of the whole amide product (**42**). Interestingly, the asymmetric 1,3-diaminopentane (**41**) was acylated by clostripain exclusively at the amino group at 1-position, whereas an acylation of the amino group at 3-position did not occur. This finding corresponds to the already mentioned observation that clostripain accepts branched amines less specifically than nonbranched derivatives. In agreement with these results, clostripain discriminates between the two primary amino groups of **41** forming exclusively the single-acylated isomer at 1-position. From a synthetic point of view, this distinct selectivity makes clostripain a useful and easy tool for selective acylations of amino components with various amino groups differing in the degree of branching without the need of additional protection and deprotection steps.

It is well-known in organic chemistry that the ratio between single and double acylation of symmetric di-

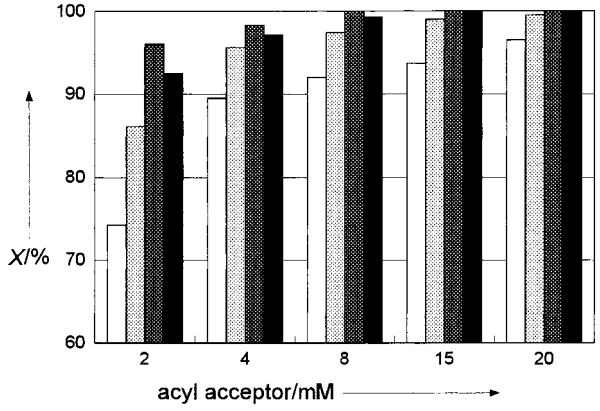


Figure 3. Influence of the chain length and concentration of the acyl acceptor on the amount of single-acylated amine *x* in clostripain-catalyzed reactions. From white to black: 1,2-diaminoethane, 1,4-diaminobutane, 1,6-diaminohexane, 1,8-diaminooctane. Conditions: 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, [acyl acceptor]$_0$ = 20 mM, [clostripain] = 5.0×10^{-7} M.

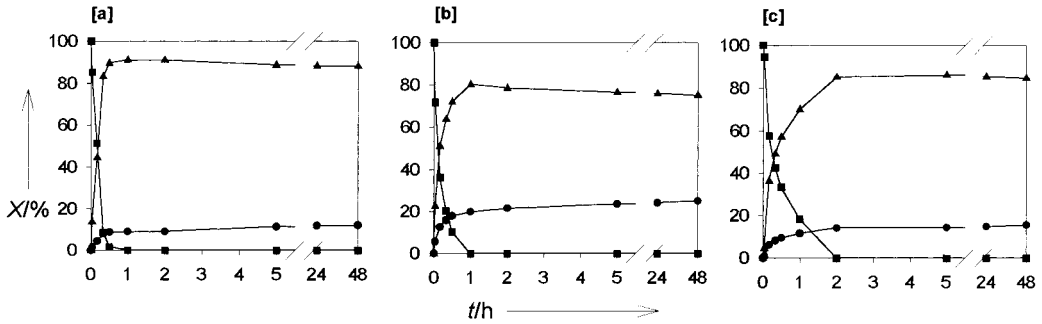


Figure 4. Course of selected clostripain-catalyzed acyl transfer reactions of Bz-Arg-OEt with several acyl acceptors: [a] pentylamine; [b] 5-aminopentanol; [c] 1,4-diaminobutane. (-■-) acyl donor Bz-Arg-Et, (-▲-) aminolysis product, (-●-) hydrolysis product. Conditions: 0.025 M $Na_2CO_3/NaHCO_3$ buffer, pH 10.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 25 °C, [acyl donor] = 2 mM, [acyl acceptor]$_0$ = 8 mM, [clostripain] = 5.0×10^{-7} M.

Table 5. Partition Values *p* of Clostripain-Catalyzed Acyl Transfer Reactions of Bz-Phe-OGp with Selected Amines and Amino Alcohols[a]

acyl donor	acyl acceptor		product		*p* value [mM]
Bz-Phe-OGp	H_2N	(79)	Bz-Phe-NH	(86)	0.05
Bz-Phe-OGp	H_2N	(80)	Bz-Phe-NH	(87)	0.15
Bz-Phe-OGp	H_2N	(81)	Bz-Phe-NH	(88)	0.41
Bz-Phe-OGp	H_2N OH OH	(82)	Bz-Phe-NH OH OH	(89)	0.88
Bz-Phe-OGp	H_2N OH	(83)	Bz-Phe-NH OH	(90)	0.10
Bz-Phe-OGp	H_2N OH	(84)	Bz-Phe-NH OH	(91)	0.43
Bz-Phe-OGp	H_2N OH	(85)	Bz-Phe-NH OH	(92)	0.17

[a] Conditions: 0.2 M HEPES buffer, pH 8.0, 0.2 M NaCl, 0.01 M $CaCl_2$, 2.5% DMF, 25 °C, [acyl donor] = 2 mM, [acyl acceptor]$_0$ = 20 mM at pH 10.0, [clostripain] = 3.3×10^{-6} M.

amines depends on the ratio of reactant concentrations. To evaluate whether this ratio also affects the enzymatic reaction, acyl transfers using various acyl acceptor concentrations were performed. Figure 3 illustrates the amount of single-acylated diamine on the whole amide product. In analogy to other chemical reactions, it is obvious that the degree of double acylation increases with a decrease of the acyl acceptor concentration. Therefore, the highest amount of double-acylated amide product was found at equimolar concentrations of both reactants. However, in contrast to chemical reactions, the enzymatic approach seems to be additionally affected by the chain length in the symmetric diamine. Whereas the short 1,2-diaminoethane shows the highest degree of double acylation, an elongation of the chain length favors the formation of a single-acylated product. Consequently, the amount of double-acylated product drops synergistically both by the increase of acyl acceptor excess and by the elongation of the chain length of the diamine.

The course of the kinetically controlled peptide synthesis by protease catalysis is typically characterized by an optimum of product formation followed by a secondary hydrolysis of the newly formed peptide bond. Therefore, the enzymatic reaction needs an accurate time control to avoid losses of peptide product. Usually, the specificity data derived from the synthesis reactions correspond to the data derived from the native hydrolysis reaction.[12] Accordingly, the high acylation rates found for the most non-amino acid-derived amines indicate a high specificity of the enzyme toward the formed sequence and, therefore, equally high rates of hydrolysis. But indeed all synthesis reactions occur practically irreversible without any secondary hydrolysis as exemplary illustrated by Figure 4. Even after reaction times of 1 and 2 days, practically no cleavage of the synthesis products occurs. This peculiarity was also found for acyl acceptors whose structure closely corresponds to that of coded amino acids, as for instance, leucinol (**26**). This behavior becomes even more remarkable since clostripain is well-known for its high amidase activity which usually results in a fast cleavage of the newly formed peptide bond.[7a] Up to now it is unclear whether this unusual "substrate"-induced effect is based

(12) (a) Fersht, A. R.; Blow, D. M.; Fastrez, J. *Biochemistry* **1973**, *12*, 2035. (b) Schellenberger, V.; Braune, K.; Hofmann, H.-J.; Jakubke, H.-D. *Eur. J. Biochem.* **1991**, *199*, 623. (c) Schellenberger, V.; Siegel, R. A.; Rutter, W. J. *Biochemistry* **1993**, *32*, 4344.

on an incorrect binding of these synthesis products or an incomplete catalysis. Since these derivatives still efficiently bind to clostripain (data not shown), this principle may be useful for the design of specific as well as selective inhibitors. From a synthetic point of view, there is no risk of any proteolytic side reactions and, therefore, no need of time control of the enzymatic synthesis reaction.

Apart from the broad nucleophile specificity described here, clostripain possesses a narrow primary specificity for arginine. Whereas the coupling of lysine at P_1-position is still possible,[7b] all other amino acids are not accepted by the enzyme. Consequently, the synthetic utility of this protease is originally limited to both of these amino acid residues. Recently, by use of 4-guanidinophenyl esters of acylated amino acid derivatives, we could show that clostripain recognizes this special type of substrates even when derived from nonspecific amino acids.[9] As a result, peptide bond formation occurs independently of the primary specificity of the enzyme. There is no doubt that the combination of both features would considerably extend the application range of this enzyme for synthesis. Therefore, model reactions with Bz-Phe-OGp as the acyl donor and selected amino components were performed. The *p* values observed for these reactions are given in Table 5. The results convincingly show that Bz-Phe-OGp acts as an efficient acyl donor acylating non-amino acid-derived amino components. Remarkably, clostripain catalyzes acylations with the nonspecific Bz-Phe-residue even more efficiently than those with the specific arginine one, as indicated by the mostly 1 order of magnitude lower *p* values. Thus, only traces of Bz-Phe-OH were formed by the enzyme. These results become still more impressive, since only small amounts of the amino components are unprotonated at the pH value of 8.0 which is dictated by the lower intrinsic stability of the guanidinophenyl ester. Therefore, only a small amount of the whole amine can serve as a deacylating component (0.2 to 3.7% depending on the individual p*K* value). This finding indicates that the strategy used for these substrate mimetics can be successfully combined with the broad tolerance of clostripain toward the acyl acceptor, realizing efficient CN-bond formations under mild pH conditions in aqueous systems.

Conclusion

The use of proteases as biocatalysts for highly selective CN-bond formations is strictly connected with the substrate specificity of these enzymes and, therefore, usually restricted toward coded amino acid residues. In contrast, the results of this study indicate a completely different behavior for the cysteine protease clostripain. The enzyme is capable of binding and acylating aliphatic amines widely varying in structure as well as amino alcohols, non-α-amino carboxylic acids, and symmetric and asymmetric diamines. Moreover, for most derivatives, acylation rates could be observed higher than those found for peptidic acyl acceptors, indicating a high flexible and relaxed S′-subsite which seems to be predominated by hydrophobic domains. Whereas the presence of polar hydroxyl groups or positively charged amino functions usually does not reduce the specificity of binding, amines with free carboxylate groups show lower reactivities. On the basis of these results, this study further provides a guideline for the application of clostripain as biocatalyst for the synthesis of peptide isosteres. Remarkably, despite the high activity of clostripain for the synthesis of peptide isosteres, the enzyme is practically inactive toward the cleavage of the newly formed amide bonds. Up to now, the molecular basis for this atypical behavior is not yet understood. In the case of substrate mimetics, unwanted proteolytic cleavages are generally prevented due to the coupling of nonspecific acyl residues. Furthermore, the remarkably high efficiency of reactions using the substrate mimetic Bz-Phe-OGp as the acyl donor demonstrated that clostripain is capable of acylating non-amino acid-derived amines with nonspecific acyl residues. Since the classical approach seriously limits the use of clostripain to reactions with Arg- and Lys-containing acyl donors, this combination considerably expands the synthetic utility of this enzyme. Based on the potential acceptance of substrate mimetics derived from non-amino acid derivatives, this approach may open up a new field of synthetic applications of proteases completely outside peptide synthesis, achieving both efficient and selective organic amide bond formations under extraordinarily mild reaction conditions.

Experimental Section

Materials. Clostripain (EC 3.4.22.8) was a gift from Fluka Chemie AG, Switzerland, and had a specific activity of 100 U mg^{-1} (1 U = amount of enzyme that hydrolyzes 1 μmol min^{-1} N^{α}-Bz-L-arginine-ethyl ester at pH 7.1 and 25 °C). The enzyme was activated before any applications for 2−3 h in the presence of 1.0 mM $CaCl_2$ containing 2.5 mM DTT. Amino acids, amines, 4-aminophenol, DCC, DMAP, benzyl chloroformate (Z-chloride), *S*-methylisothiourea, and 4-toluenesulfonic acid were purchased from commercial suppliers. All reagents were of the highest available commercial purity. Solvents were purified and dried by the usual methods. Mass spectra were recorded using thermospray ionization or by MALDI-ToF.

Chemical Syntheses. Bz-Phe-OGp was prepared by condensation of Boc-Phe-OH and 4-[*N*′,*N*″-bis(Z)guanidino]phenol following the procedure described by Sekizaki et al.[13] Because of the more advantageous preparation and higher reactivity, *N*,*N*′-bis(Z)-*S*-methylisothiourea was used for the amidination of 4-aminophenol to synthesize 4-[*N*′,*N*″-bis(Z)guanidino]phenol[14] instead of 1-[*N*,*N*′-bis(Z)amidino]pyrazole. The benzoyl-protected ester was prepared, starting with the deprotection of the N^{α}-amino group with trifluoroacetic acid and subsequent benzoylation using benzoyl chloride. A final catalytic hydrogenation of the bis(Z)-protected ester results in the 4-*N*′,*N*″-deprotected 4-guanidinophenyl ester. The amino acid methyl esters were synthesized following the procedure described by Brenner et al.[15] The amino acid amides were prepared from the appropriate methyl esters by ammonolysis with ammonia. The identity and purity of all final products were checked by analytical HPLC (220 nm), NMR, thermospray mass spectroscopy, and elemental analysis. In all cases, satisfactory elemental analyses data were found (±0.4% for C, H, N).

Enzymatic Syntheses. Enzymatic reactions with Bz-Arg-OEt were performed in 0.025 M Na_2CO_3/$NaHCO_3$ buffer, pH 10.0 containing 0.2 M NaCl and 0.01 M $CaCl_2$ at 25 °C. Reactions with Bz-Phe-OGp were performed in 0.2 M HEPES buffer, pH 8.0, containing 0.2 M NaCl, 0.01 M $CaCl_2$, and 2.5% DMF at 25 °C. Stock solutions of acyl donor esters (4 mM) were prepared in distilled water. In the case of Bz-Phe-OGp, 5% DMF was added to realize a complete solubility of the ester. Amino components were dissolved in 0.4 M HEPES buffer (pH 8.0) or 0.05 M Na_2CO_3/$NaHCO_3$ buffer (pH 10.0) containing

(13) Sekizaki, H.; Itoh, K., Toyota, E.; Tanizawa, K. *Chem. Pharm. Bull.* **1996**, *44*, 1577.
(14) Lal, B.; Gangopadhyay, A. K. *Tetrahedron Lett.* **1996**, *37*, 2483.
(15) Brenner, M.; Müller, H. R.; Pfister, R. W.; *Helv. Chim. Acta* **1950**, *33*, 568.

0.4 M NaCl and 0.02 M $CaCl_2$. To neutralize hydrochlorides or hydrobromides, appropriate equivalents of NaOH were added to the stock solutions of the amino components. If not otherwise stated, the final acyl donor and nucleophile concentration was 2 mM and 20 mM, respectively. The concentration of the amino components was calculated as free, N^{α}-unprotonated nucleophile concentration $[HN]_0$ according to the formalism of Henderson−Hasselbalch $[HN]_0=[N]_0/(1+10^{pK-pH})$. For the reactions with Bz-Phe-OGp, the amines were used in identical concentrations as used for reactions with Bz-Arg-OEt. In these cases the actual concentration of $[HN]_0$ within the reaction mixture was calculated to the lower pH value of 8.0. After thermal equilibration of the assay mixtures, the reactions were started by addition of the enzyme, resulting in active enzyme concentrations of 5.0×10^{-7} M for reactions with Bz-Arg-OEt and 3.3×10^{-6} M in the case of Bz-Phe-OGp. Reaction times of 10−120 min led to a complete ester consumption. For the HPLC analysis aliquots were withdrawn and diluted with a quenching solution of 50% aqueous methanol containing 1% trifluoroacetic acid. For each acyl donor and acyl acceptor, an experiment without enzyme was carried out for determining the extent of spontaneous ester hydrolysis which was strictly less than 5%. On the basis of the same control experiments, nonenzymatic aminolysis of the acyl donor esters was investigated and could be ruled out. The results reported are the average of at least three independent experiments. The identity of the formed peptide and amide products was validated by thermospray and MALDI-ToF mass spectroscopy, respectively. NMR measurements were used for the examination of the enzymatically formed amide bonds.

HPLC Analyses. Samples were analyzed by analytical reversed phase HPLC on C18 polymer coated columns [Vydac 218TP54, 5 μm, 300 Å, (25 × 0.4 cm), and Grom Capcell, 5 μm, 300 Å, (25 × 0.4 cm)] and a C4 reversed phase column [Vydac 214TP, 10 μm, 300 Å, (25 × 0.4 cm)] which were thermostated at 25 °C and eluted with various mixtures of water/acetonitrile containing 0.1% trifluoroacetic acid under gradient conditions. Detection was at 254 nm to monitor the aromatic chromophores of the acyl donors. Thus, the yields could be determined from the peak areas of the hydrolysis and aminolysis products, whereby 4-toluenesulfonic acid served as an internal standard. In the case of chromophoric amino components, the yields were calibrated from the lack of hydrolysis products by at least five independent experiments.

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