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Subtilisin-Catalyzed Synthesis of Amino Acid and Peptide Esters. Application in a Two-Step Enzymatic Ligation Strategy

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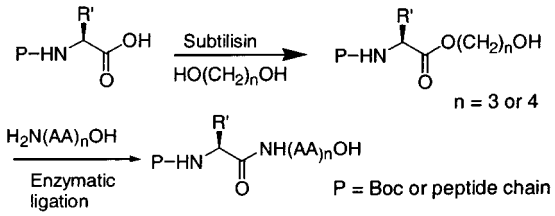
ABSTRACT



We describe an efficient enzymatic approach to the synthesis of amino acid and peptide esters. The serine protease subtilisin Carlsberg (EC 3.4.21.62) was found to efficiently catalyze the specific formation of Cα-carboxyl 3-hydroxypropyl or 4-hydroxybutyl esters of certain Boc-amino acids and peptides in high-content 1,3-propanediol or 1,4-butanediol solution, with substrate specificity parallel to that of the normal hydrolytic reaction. This approach can be coupled with kinetic-control reverse proteolysis in a two-step enzymatic peptide ligation scheme.

Amino acid and peptidyl Cα-esters are important and versatile intermediates in organic and peptide synthesis. Such esters are widely used for temporary protection of the α-COOH groups,[1] and more importantly they can also be transformed to a variety of functionalities[2] such as amide, hydrazide, or aldehyde for further transformation to form more complex structures. A particularly prominent application of these esters resides in their use as acyl donors in kinetically controlled enzymatic peptide synthesis.[3] Simple amino acid esters are traditionally prepared by chemical means through either strong acid catalysis or intervention of a dehydration agent. At the same time, special resins can be developed for preparing peptide esters by solid-phase synthesis.[4,2c−d,3f−g] Here, we report an alternative strategy of using a simple and mild enzymatic method for the preparation of amino acid or peptide Cα-esters. Although serine or cysteine proteases are very efficient in catalyzing the hydrolysis of peptide Cα-esters, more so than in the hydrolysis of the respective peptide bonds, the reverse esterase activity of such proteases for ester synthesis has seldom been exploited. One pioneer work was Kise's investigation on the use of α-chymotrypsin and subtilisin for the esterification, with poor to moderate efficiency, of simple Nα-protected aromatic amino acids in high-content ethanol solution.[5] We found that the bacterial serine protease subtilisin Carlsberg retained high catalytical

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activity in two aliphatic diols, 1,3-propanediol and 1,4-butanediol. In the presence of this enzyme, many Boc-amino acids and unprotected peptides, when solubilized in 1,3-propanediol or 1,4-butanediol containing 1−2.5% H_2O, were selectively esterified at their C^{α}-COOHs to the corresponding diol mono-esters in excellent yields (Table 1, see also Supporting Information for more examples).

Table 1. Subtilisin-Catalyzed Esterification of Boc-amino Acids and Peptides

$$R_1CO\text{-}OH + R_2\text{-}OH \underset{}{\overset{\text{Subtilisin}}{\rightleftharpoons}} R_1CO\text{-}OR_2 + H_2O$$

no.	substrate R_1CO-OH	alcohol R_2-OH	time (h)	yield[a] (%)	ester MS[b]
1	Boc-F-OH	HOPrOH	10	85	324
2	Boc-F-OH	HOBuOH	10	90	338
3	Boc-F-OH	EtOH	10	22	294
			48	65	
4	Boc-D-F-OH	HOPrOH	24	0	
5	Boc-Y-OH	HOPrOH	9	91	340
6	Boc-M-OH	HOPrOH	39	94	308
7	Boc-L-OH	HOPrOH	24	95	290
8	Ac-FA-OH	HOPrOH	60	86	337
9	neurotensin	HOPrOH	2.5	90	1731.3
10	xenopsin	HOBuOH	3	92	1166.2
11	neuropeptide	HOBuOH	3	66	1198.4
12	endothelin	HOBuOH	72	80	2565.5

[a] Yields were calculated from HPLC. [b] $[M + H]^+$ mass data were obtained with FAB-MS for entries 1−8 and MALDI-MS for entries 9−12. Amino acids in entries 1−8 are represented by the single-letter code. HOPrOH: 1,3-propanediol. HOBuOH: 1,4-butanediol. Water content in the reaction was 2.5% for entries 1−7 and 2% for entries 8−12. Enzyme/sustrate molar ratio was 1/500. Amino acid concentration was 50 mM. Peptides were used at 20 mM (10 mM for endothelin). Neorotensin: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH. Xenopsin: H−Phe-His-Pro-Lys-Arg-Pro-Trp-Ile-Leu-OH. Neuropeptide (The Head Activator): pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH. Endothelin: H−Cys-Ser-Cys-Ser-Ser-Leu-Met-Asp-Lys-Glu-Cys-Val-Tyr-Phe-Cys-His-Leu-Asp-Ile-Ile-Trp-OH (1−4 and 2−3 disulfide). Peptides 9−12 were used in TFA salt forms.

The enzymatic activity of subtilisin in the two diols was found to be much higher than in ethanol when the reactions of Boc-Phe-OH in these three alcohols were compared. In addition, the reaction was stereospecific, as Boc-D-Phe-OH was totally unreactive (Table 1, entry 4). Subtilisin retained its broad substrate specificity of favoring amino acids with aromatic, long aliphatic, or neutral side chains at the P1 position. The esterification was also regiospecific at the C-terminal α-COOH for the tested peptides, with the side chain carboxylic groups of Asp or Glu unaffected. This was confirmed by enzymatic digestion of the esterified Glu/Asp-containing peptides using a specific endopeptidase, V_8, which can only hydrolyze a Glu/Asp-Xxx peptide bond. For example, for the peptide neurotensin (Table 1, entry 9) which contains a Glu at position 4, V_8 treatment of its ester product in 50 mM phosphate buffer (pH 7.8) gave two fragments corresponding respectively to pGluLeuTyrGlu-OH and H-AsnLysProTyrIleLeu-OPrOH as confirmed by MS analysis. Similar results were obtained with V_8 digestion of endothelin hydroxybutyl ester (Table 1, entry 12).

Many unprotected peptides were found to be readily soluble in these two diols,[6] and their esterification proceeded smoothly without significant cleavage (through hydrolysis or alcoholysis) of the internal peptide bonds (Figure 1). In

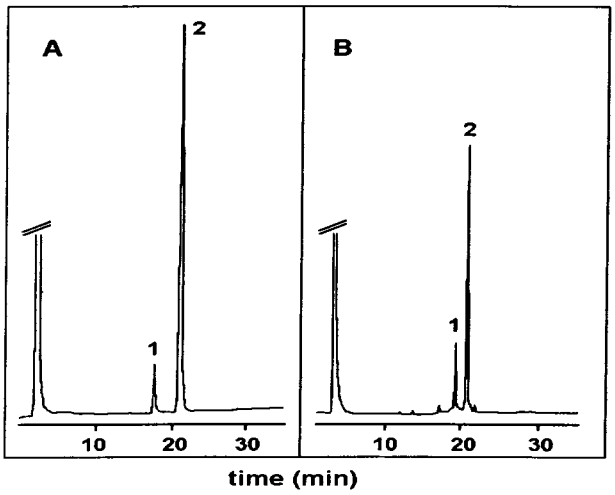

Figure 1. HPLC monotoring of the esterification of xenopsin (3 h) and endothelin (72 h) in 1,4-butanediol. (A) Peak 1, xenopsin; peak 2, xenopsin-OBuOH. (B) Peak 1, endothelin; peak 2, endothelin-OBuOH. HPLC was run on a Shimadzu system with a Vydac C18 analytical column (0.46 × 25 cm, 5 μm) at a flow rate of 1.5 mL/min, using linear gradient of 20−55% for 35 min of buffer B (60% CH_3CN in H_2O, 0.04%TFA) in buffer A (5% CH_3CN in H_2O, 0.045% TFA).

fact, certain peptides (Table 1, entries 9−11) displayed even higher reactivity than simple amino acids. In general, 1,3-propanediol is a better solvent for these peptides, but 1,4-butanediol has a practical advantage of generating ester products that are more separable by HPLC from the starting materials. Substrate solubility is clearly one of the factors that would limit the use of this method, especially when larger, hydrophobic peptides are concerned. One should note that the peptides examined in this report are not particularly large in size. The general use of this methodology is also limited by the substrate specificity of subtilisin, which requires certain preferred amino acids at the P1 position. In fact, nonpreferred substrates such as Boc-Val-OH and Boc-Gly-OH were not esterified after 5 days of reaction (see Supporting Information).

A plausible explanation for the catalytic activity of subtilisin in these nearly pure diols is found in nonaqueous enzymology.[7] It has been postulated that as long as an enzyme can retain a layer or so of water molecules on its surface for maintaining its catalytically active conformation,

(6) The solubility of a peptide in the two diols was found to depend on its hydrophilicity. In general, hydrophilic peptides are more soluble in these diols than hydrophobic ones. Poorly soluble hydrophobic peptides gave poor results in esterification.

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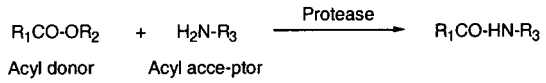
Table 2. Protease-Catalyzed Aminolysis of Esters for Peptide Ligation



						product $[M + H]^+$	
no.	acyl donor (mM)	acyl acceptor (mM)	enzyme[a]	time (min)	yield[b] (%)	calcd	found[c]
1	Ac-Phe-Ala-OPrOH (25)	H-Leu-NH_2 (200)	papain[d]	10	95[f]	390.2	390.0
2	Ac-Phe-Ala-OPrOH (25)	FI-6 (30)	papain[d]	20	66[f]	1028.2	1028.0
3	neuropeptide-OBuOH (2)	RF-9 (6)	subtiligase[e]	10 (h)	80	2221.0	2222.5

[a] Enzymes were used at a 1/500 molar ratio to acyl donors. [b] Yields were calculated from HPLC on the basis of acyl donors. [c] Mass spectral data were obtained with FAB-MS for entry 1 and MALDI-MS for entries 2−3. [d] Reaction was run in 60% acetonitrile in 0.3 M Triethanolamine buffer (pH 8.5) and [e]in aqueous Tricine buffer (0.1M, pH 8.0) containing 2 mM DTT at 37 °C. [f] In these two cases, ester hydrolysis accounted for the rest of percentage. FI-6: H-Phe-Val-Phe-Asn-Lys-Ile-OH. RF-9: H-Arg-Tyr-Gln-Ala-Glu-Val-Ser-Leu-Phe-OH.

the remaining bulk aqueous milieu is replaceable with an organic solvent. Thus, in our proposed system, subtilisin captures the essential amount of water after distribution of the 1−2.5% of H_2O between the hydrophilic enzyme surface and the diol solvent. The yield of esterification was decreased when higher water content was used, due to a shift of the reaction equilibrium to the reactant direction (Table 1, reaction scheme). These diol systems effectively lowered the amidase activity of subtilisin, since no significant cleavage of the internal peptide bonds was observed in our peptide examples which contain one or more susceptible peptide bonds to subtilisin under normal hydrolytic conditions. This is in general agreement with previous observations that a high content of organic solvents such as DMF or DMSO diminishes or suppresses the amidase activity of serine or cysteine proteases while retaining their esterase activity at a significant level.[3e]

A number of applications can be envisioned for these particular ester products. For instance, after the oxidation of the free hydroxyl to an aldehyde or carboxyl, the amino acid esters may find their use as new chiral synthons in organic synthesis. As part of our efforts in developing peptide ligation strategies, we envisioned that this enzymatic esterification method could be coupled with kinetic-control enzymatic aminolysis of peptide esters in a two-step enzymatic peptide ligation scheme depicted in the abstract graphic. We tested the feasibility of this strategy by using some of these esters as acyl donors for enzymatic peptide synthesis. As shown in Table 2, several peptides were prepared in satisfactory yields through catalysis of different proteases. A particularly noteworthy point was the use of subtiligase, a DNA-recombinant engineered subtilisin designed for enzymatic segment ligation,[3g] in the synthesis of a 20-residue peptide using a 11-mer peptide ester (from Table 1, entry 11) prepared by the above method as the acyl donor. In this example, ligation took place smoothly for 10 h in a normal aqueous buffer at relatively low molar concentrations of both components and no significant hydrolysis of the ester linkage or peptide bond was observed. Our two-step enzymatic ligation scheme is conceptually similar to a method reported by Hwang et al. who have utilized a chemical method to activate the α-carboxylic group to 5(4*H*) oxazolone in place of carboxyl esters as activated acyl donors for enzymatic peptide synthesis.[8] These enzymatic approaches represent an important supplement to the currently available chemical ligation methods.[9,10]

In conclusion, we have shown that certain Boc-protected amino acids and peptides can be selectively esterified by making use of the reverse esterase activity of subtilisin, even in the presence of other unprotected functionalities including the equally reactive side chain carboxylic groups. In addition, such esters are useful substrates as acyl donors for kinetically controlled enzymatic peptide synthesis. Our proposed racemization-free esterification system provides a useful alternative to the conventional chemical methods for ester synthesis, which usually require harsh and stringent reaction conditions. These results may stimulate further research interest in the design and development of environmentally benign processes for the manufacturing of agriculturally or therapeutically important products.

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Supporting Information Available: Detailed experimental procedures, HPLC profiles, and ^1H NMR data of certain Boc-amino acid esters. This material is available free of charge via the Internet at http://pubs.acs.org.

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