*J. Enzyme Inhibition*, 1991, Vol. 4, pp. 289–298
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# INHIBITION OF PROTEASES WITH ENKEPHALIN-ANALOGUE INHIBITORS

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*(Received 17 November 1989)*

*N*-peptidyl-*O*-acyl hydroxylamines have proven to be effective and selective mechanism-based inhibitors of serine and cysteine proteases as demonstrated using enzymes with specificities for hydrophobic amino acids at the cleavage site[1–6]. Here, we report for the first time the inhibition of proteases able to accommodate cationic amino acid side chains in their binding pockets using compounds of this inhibitor class. Trypsin and papain are inactivated by enkephalin-analogue diacyl hydroxylamines in a time-dependent and irreversible manner exhibiting second-order rate constants in the range of 100–1000 $M^{-1} \cdot s^{-1}$. In contrast, human cerebrospinal fluid dynorphin-converting enzyme (hCSFDCE) is inhibited only moderately by these inhibitors. Mechanistic implications have been derived.

KEY WORDS: Protease, mechanism-based inhibitor, papain, trypsin, dynorphin-converting enzyme, diacyl hydroxylamine, enkephalin, mechanism.

## INTRODUCTION

Serine and cysteine proteases with trypsin-like substrate specificity are known to be processing enzymes in physiological reaction cascades. They selectively activate or deactivate regulatory peptides, binding proteins or enzymes by limited proteolysis[7]. In recent years increasing attention has been given to enzymes with similar properties but especially those involved in neuropeptide and peptide hormone processing[8]. Some of these enzymes, among them the dynorphin converting enzymes, characterized as serine are known for their high selectivity for peptide linkages consisting of two cationic amino acids (for a recent review see reference 9). To analyze the regulatory activity of such proteases at the cellular level and to gain information on mechanistic properties, specific inhibitors would be a valuable tool.

*N*-peptidyl-*O*-acyl hydroxylamines are mechanism-based inhibitors of serine and cysteine proteases[1–6] and have been applied successfully in biological studies[10–12]. They are likely to react by enzyme-induced decomposition at the –NH–O–linkage[2,3]. During nucleophilic attack of the target enzyme on the scissile carbonamide bond, a leaving nucleofuge (the *O*-acyl residue) leaves behind either a highly electrophilic nitrene intermediate or (after Lossen-rearrangement) a reactive isocyanate, which covalently modifies the active site of the attacking enzyme (Scheme I). Bearing in mind this inhibition principle we have designed appropriate enkephalin-analogue structures

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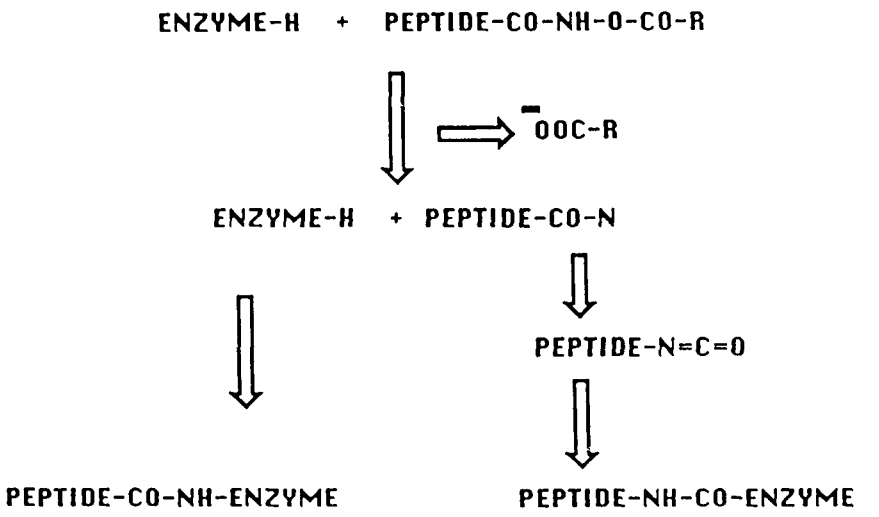


SCHEME 1 Hypothetical mechanism for reaction of diacyl hydroxylamines with serine and cysteine proteases.

as potential inactivators of enzymes exhibiting trypsin-like substrate specificity including hCSF dynorphin converting enzyme.

## MATERIALS AND METHODS

### *Inhibitors and substrates*

The enkephalin-analogue *N*-peptidyl-*O*-benzoyl hydroxylamines were synthesized as described in earlier work[1-3]. Briefly, BOC-Tyr-Gly-Phe-Leu-Lys-OMe was obtained by coupling of BOC-Tyr-Gly-Gly-Phe-Leu-OH (Bachem, Bubendorf, Switzerland) and H-Lys (ε-BOC)-OMe (gift of Dr. D. Brömme, Department of Biochemistry, Martin-Luther-University of Halle) applying the mixed-anhydride method. BOC-Tyr-Gly-OMe was obtained by the same method using BOC-Tyr-OH (Bachem) and H-Gly-OMe (student preparation) as starting materials. The methyl esters were converted to the *N*-peptidyl hydroxamic acids by methanolic hydroxylamine solution and were converted to *N*-peptidyl-*O*-benzoyl hydroxylamines using benzoyl chloride (Merck, Darmstadt, F.R.G.) in subsequent Schotten-Baumann reactions. The BOC-protecting groups were removed using HCl/glacial acetic acid to yield the appropriate hydrchlorides. Purity and molecular composition were verified by reverse-phase HPLC and FAB-mass spectrometry. Table I lists the properties of the obtained products.

All reagents used for synthesis were of the highest purity available and organic solvents were dried before use. Benzoyl-DL-Arginine-pNitroanilide (BANA; Sigma, St. Louis, U.S.A.) was used as chromogenic substrate in uv-spectrometric analysis of the enzyme activities and in the inactivation studies. Dynorphin A used in radioimmunoassays and HPLC-product analysis was purchased from Bachem (Bubendorf, Switzerland), dynorphin B was prepared by Dr. G. Lindeberg, Department of Im-

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TABLE I
Parameters and properties of *N*-peptidyl-*O*-benzoyl hydroxylamines

| Compound | Formula | M.W. | FAB/MS* | Elution time (min)** |
|---|---|---|---|---|
| 1. Leu-enk-Lys$^6$-NHO-Bz | $C_{44}H_{53}N_8O_9$ | 801.92 | 803 | 27 |
| 2. BOC-Tyr-Gly-NHO-Bz | $C_{23}H_{27}N_3O_7$ | 457.48 | 458 | 34 |
| 3. Tyr-Gly-NHO-Bz | $C_{18}H_{18}N_3O_5$ | 356.36 | 358 | 16 |

* Molecular peak $MH^+$ estimated by fast atom bombardment mass spectrometry (FAB/MS) (see Materials and methods).

** Reversed-phase HPLC; gradient acetonitrile/$H_2O$: 1. 15–50% (30 min), 2. 20–70% (50 min), 3. 20–50% (35 min)

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munology, University of Uppsala (Sweden) and Leu-enkephalin-Arg$^6$ was from Peninsula Laboratories (Belmont, U.S.A.). For the assays (see below) stock solutions of inhibitors and substrates were prepared in dimethylsulfoxide (DMSO) or in distilled water. In order to investigate the concentration dependence of the enzyme inhibition these solutions were further diluted with water. In no case was the concentration of organic solvent higher than 2.5% (v/v). TRIS and HEPES buffer substances were from Sigma (St. Louis, U.S.A.), EDTA and cysteine were from Merck (Darmstadt, F.R.G.). Papain (type IV) was from Sigma (St. Louis, U.S.A.) and trypsin (twice crystallized) was purchased from Serva (Heidelberg, F.R.G.).

*Enzymes, assays and product analysis*

All inactivation studies using these enzymes were performed at 30°C in 50 mM HEPES-buffer pH 7.5 using BANA as substrate. In the papain assay, the buffer solution contained 5 mM cysteine and 2 mM EDTA. The enzyme was activated for at least 5 min in the solution and reactions were started by adding substrate to the mixture.

The absorbance change at 390 nm during enzyme-catalyzed substrate hydrolysis was monitored on an Ultrospec K kinetics spectrophotometer (LKB Biochrom, Cambridge, England) equipped with the Autofill K flowcell compartment and a Peltier heating/cooling element for temperature control. Kinetic data were printed after each run and analyzed using nonlinear regression programs running on a Multitech PC-compatible computer. The specific amidase activity under the above conditions (applying 1.0 mM substrate) was 0.3 U/mg and 1.03 U/mg for papain and trypsin, respectively. Rate constants of inactivation were evaluated using computational methods extensively described and discussed in earlier work[3,4].

Dynorphin-converting enzyme was purified from human cerebrospinal fluid as described before[13]. The enzymatic activity was monitored by measuring the formation of Leu-enkephalin-Arg$^6$ from synthetic dynorphin B using a radioimmunoassay (RIA) specific for the product or by HPLC chromatography as essentially described in previous work[13,14]. Preincubation of the enzyme with inhibitors was performed at 37°C for 30 min. The enzyme assay was initiated by addition of dynorphin B solution. The typical assay volume of 40 $\mu$l contained 50 mM TRIS-HCl buffer, pH 7.8, 1.0 mg enzyme and 1.6 mM dynorphin B as substrate. Incubation was performed in Eppendorf tubes at 37°C for 60 min. The specific activity of hCSF DCE was estimated to be 0.4 mU/mg protein.

Product and structural analysis of the newly synthesized compounds and the

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reaction products of their thermal degradation in the absence or presence of enzyme was conducted on a LKB-2150/52 HPLC-system equipped with a 2140 Rapid Spectral Photometer and a Multitech PC-compatible computer for data acquisition[15] and handling using a RP-Ultropac column, TSK ODS-120 T (LKB, Bromma, Sweden).

The linear elution gradient (for run-times and concentrations see legends and text) was made in all cases from acetonitrile/water containing 0.04% TFA. 0.5 ml samples were collected on a Gilson model 201 fraction collector and dried in a Savant Speed vacuum concentrator before mass spectroscopic analysis.

Mass spectra were taken in positive mode on a Finnigan Mat 90 double focussing instrument (Bremen, F.R.G.), operating at a 5 kV accelerating voltage. Xenon was used as a source of primary beam (Ion Tech saddle field gun), adjusted to 7 kV and 2 mA. Samples were redissolved in methanol, mixed with an equal amount of glycerol matrix and approximately 1.0 nmole of substance was analyzed. Magnetic scan was chosen at a rate 5 s/decade and data were acquired and processed by a PDP-11 minicomputer system.

## RESULTS AND DISCUSSION

Incubation of trypsin and papain with enkephalin-analogue *N*-peptidyl-*O*-benzoyl hydroxylamines resulted in rapid inactivation of the target enzymes.

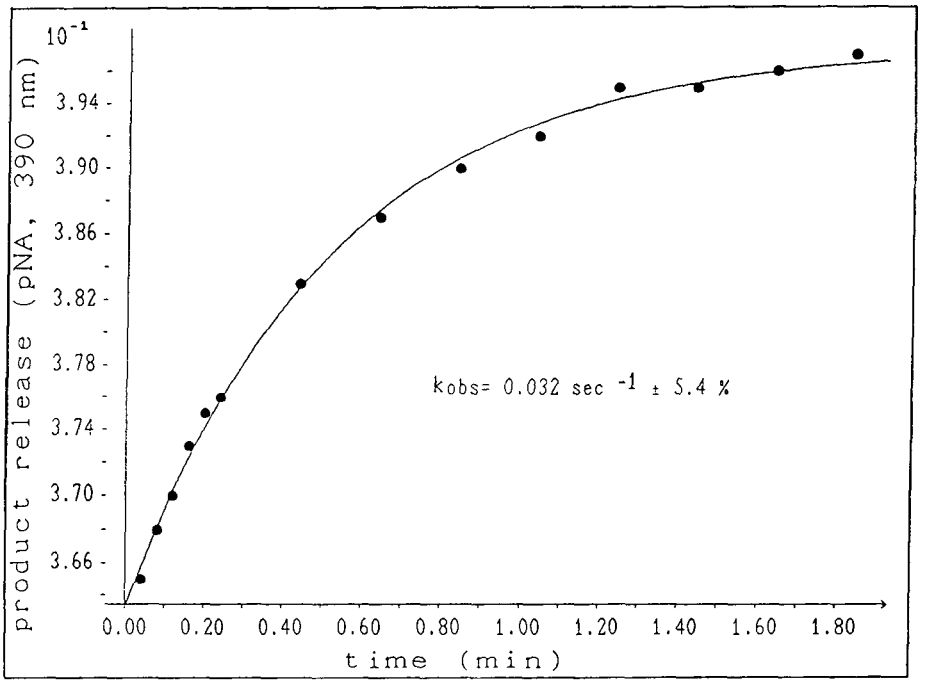


FIGURE 1 Inactivation of papain (0.046 mM) in presence of substrate (BANA, 1.0 mM) and inhibitor (Leu-enk-Arg$^6$-NHO-Bz, 0.12 mM).

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This reaction was studied according to the method of Tsou[16] in the presence of a substrate using the competition of substrate and inhibitor for the enzyme's binding site to estimate the rate constants. Since the decrease of enzyme concentration during incubation with inhibitor follows pseudo-first order kinetics the enzyme-catalyzed substrate turnover occurs proportionally. Applying chromogenic substrates and establishing steady-state conditions during the inactivation time, first-order rate constants may be obtained by fitting absorbance and time values to an exponential function (for a sample run see Figure 1). Measurements at constant inhibitor and different substrate concentration give different $k_{obs}$-values. Extrapolation of these values results in the rate constant at a substrate concentration of zero ($k_{app}$). By fitting these rate constants evaluated at different inhibitor concentrations to a hyperbola, the inactivation parameters $K_i$ and $k_{inact}$ may be obtained (Figure 2).

Papain, a cysteine protease with very broad substrate specificity, is inactivated by all three compounds, while trypsin, a serine protease exclusively specific for cationic amino acids in the $P_1$-position of its substrates, is inactivated accordingly only by Leu-enk-Lys[6]-NHO-Bz (Table II).

In contrast, prolonged incubation (30 min) of hCSF DCE with the inhibitors applied in concentrations as high as 1.0 mM, resulted only in weak inhibition of this target enzyme (Figure 3). This result is in agreement with previous studies, in which we have screened the potential inhibitory activity of 12 different *N*-peptidyl-*O*-acyl hydroxylamines against hCSF DCE, a serine enzyme, and human spinal cord DCE,

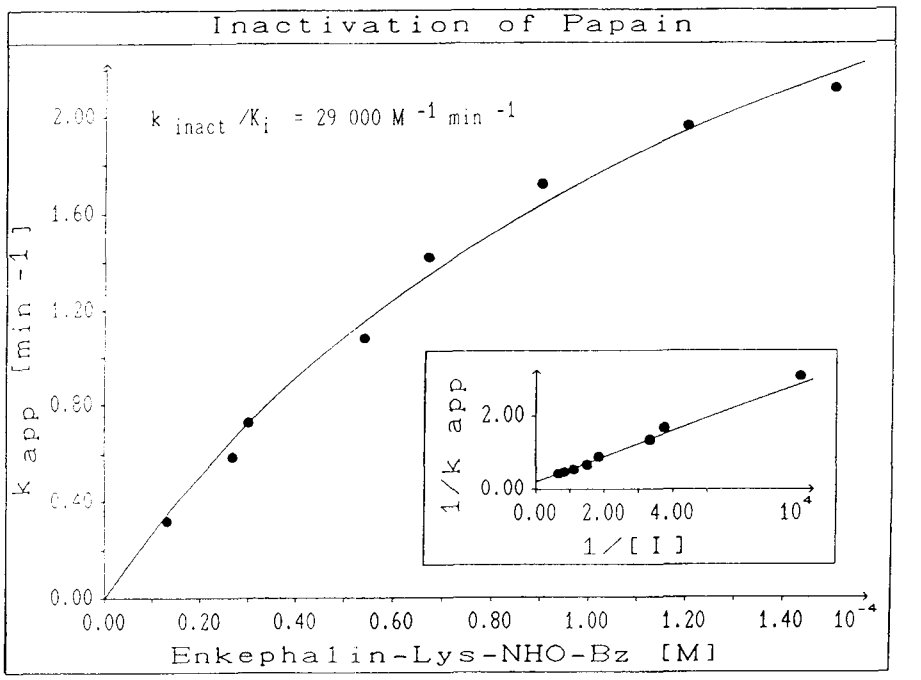


FIGURE 2 Dependence of the inactivation rate constants ($k_{app}$) on the inhibitor concentration during reaction of papain with Leu-enk-Lys[6]-NHO-Bz. (For data processing and parameter evaluation see Materials and methods)

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TABLE II
Inactivation of trypsin and papain with diacyl hydroxylamines*

| Compound | Trypsin $k_{inact}$ ($s^{-1}$) | Trypsin $K_i$ (mM) | Trypsin $k_{inact}/K_i$ ($M^{-1} \cdot s^{-1}$) | Papain $k_{inact}$ ($s^{-1}$) | Papain $K_i$ (mM) | Papain $k_{inact}/K_i$ ($M^{-1} \cdot s^{-1}$) |
|---|---|---|---|---|---|---|
| 1. | 0.017 | 0.22 | 77 | 0.073 | 0.149 | 490 |
| 2. | | n.i.** | | 0.056 | 0.91 | 642 |
| 3. | | n.i.** | | | | 399*** |

* inhibitor concentrations: (1) 0.15–0.013 mM; (2) 0.1–0.02 mM; (3) 0.2–0.04 mM; for measurement conditions see Materials and methods
** n.i.: no inactivation observed during 30 min incubation with 0.5 mM inhibitor
*** no complex formation observed in the inhibitor concentration range used

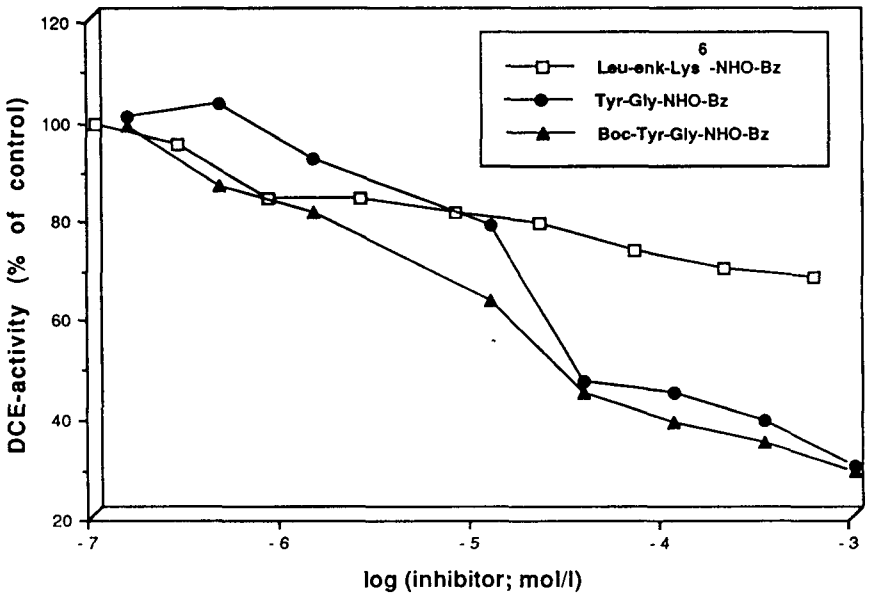


FIGURE 3 Activity (% of control) of hCSF DCE depending on the concentration of enkephalin-analogue diacylhydroxylamines. (Activity estimation using a RIA specific for Leu-enk-Arg$^6$, see Materials and methods)

a cysteine enzyme[17,18]. In both cases, only reversible inhibition was observed with the inhibitors, which inactivate other proteases with second-order rate constants of more than $1 \times 10^6\ M^{-1} \cdot s^{-1}$.

According to the proposed inhibition mechanism by *N*-peptidyl-*O*-acyl hydroxylamines, nucleophilic attack on the scissile peptide bond is a prerequisite for the observed mechanism-based enzyme inactivation[2,5]. Thus, if the compounds do not match the substrate or product structure of natural peptide substrates of the target enzymes and are not productively bound, no inactivation occurs[1-6]. For instance, according to the substrate specificity of the proline-specific dipeptidyl peptidase IV, potential inhibitors having D-amino acids in $P_1$- or $P_2$-position of their sequence do not affect the enzyme's activity[19].

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TABLE III
Decomposition rate-constants for enkephalin-analogue diacyl hydroxylamines*

| Compound | k ($s^{-1}$) (+/− s.e.m. (%)) | FAB/MS** |
|---|---|---|
| 1. Leu-enk-Lys$^6$-NHO-Bz | $6.5 \times 10^{-4}$ (0.8) | 699 |
| 2. BOC-Tyr-Gly-NHO-Bz | $1.6 \times 10^{-5}$ (7.8) | 354 |
| 3. Tyr-Gly-NHO-Bz | $1.9 \times 10^{-5}$ (4.9) | 253 |

* Incubation in 50 mM TRIS-HCl buffer, pH 7.8, at 37°C, registration of absorbance decrease at 235 nm due to the release of benzoic acid[2], compare Materials and methods

** Molecular peak, $MH^+$, of the products formed, estimated by fast atom bombardment mass spectrometry (FAB/MS). Values correspond to the appropriate peptidyl hydroxamic acids.

However, in this study, the inhibitor Leu-enk-Lys$^6$-NHO-Bz, exhibiting the product-analogue structure closest to natural DCE-conversion products, was found to be the poorest hCSF DCE inhibitor.

Since spontaneous decomposition or enzyme-catalyzed inhibitor hydrolysis by the target enzyme might not be excluded[2,3], we analyzed the stability of the inhibitors under assay conditions in the presence and absence of hCSF DCE using RP-HPLC, mass spectrometry and uv-spectroscopy. Table II lists the pseudo-first order rate constants of spontaneous degradation of the enkephalin-analogue diacylhydroxylamines to peptidyl hydroxamic acids and benzoic acid. The formation of these products is in complete agreement with previous results[2,3].

The mechanism of the thermal degradation is most likely the α-elimination of the nucleofuge acyl residue[12]. In contrast to the short enkephalin-analogues, Leu-enk-Lye$^6$-NHO-Bz decomposes rather quickly. To verify this result, the compound (0.36 mM) was incubated in 50 mM TRIS-HCl buffer, pH 7.8, at 37°C. At 10 min intervals, 40 μl aliquots of the reaction mixture were withdrawn and analyzed at a RP-HPLC column (Figure 4). Calculation of the amount of product formed from the chromatograms and subsequent numeric analysis resulted in a first-order rate constant of $6.0 \times 10^{-4}\ s^{-1}$ (± 30%) which is in agreement with the constant obtained by uv-spectroscopy (compare Table III). The product was collected, dried and subjected to mass spectroscopic analysis. The resulting molecular ion peak of 699 corresponds to the molecular weight of the expected Leu-enk-Lys$^6$ hydroxamic acid[20]. Also, twenty hours incubation of the inhibitor in the presence of hCSF DCE resulted neither in different decomposition kinetics nor in another reaction product which proves clearly that hCSF DCE does not hydrolyze Leu-enk-Lys$^6$-NHO-Bz or Leu-enk-Lys$^6$-NHOH.

This result is completely different from the data obtained with other types of serine and cysteine proteases[2-6] including trypsin and papain. Additionally, it is a well known fact that peptidyl hydroxamic acids are hydrolyzed very rapidly by proteolytic enzymes[21].

Hypothetically, the observed weak enzyme-inhibitor interactions could be due to the following:

(i) The compounds lack a prolonged C-terminal peptide part (mimicking the natural substrates dynorphin A and B) which could be essential for proper binding and action of the catalytic machinery of the enzyme.

(ii) Since the –NH–O– linkage is deprotonated at neutral pH-values[2,20], the negative charge, reducing the electrophilic nature of the scissile carbonyl group, might prevent productive binding and nucleophilic attack necessary to release an inactivating nitrene or isocyanate.

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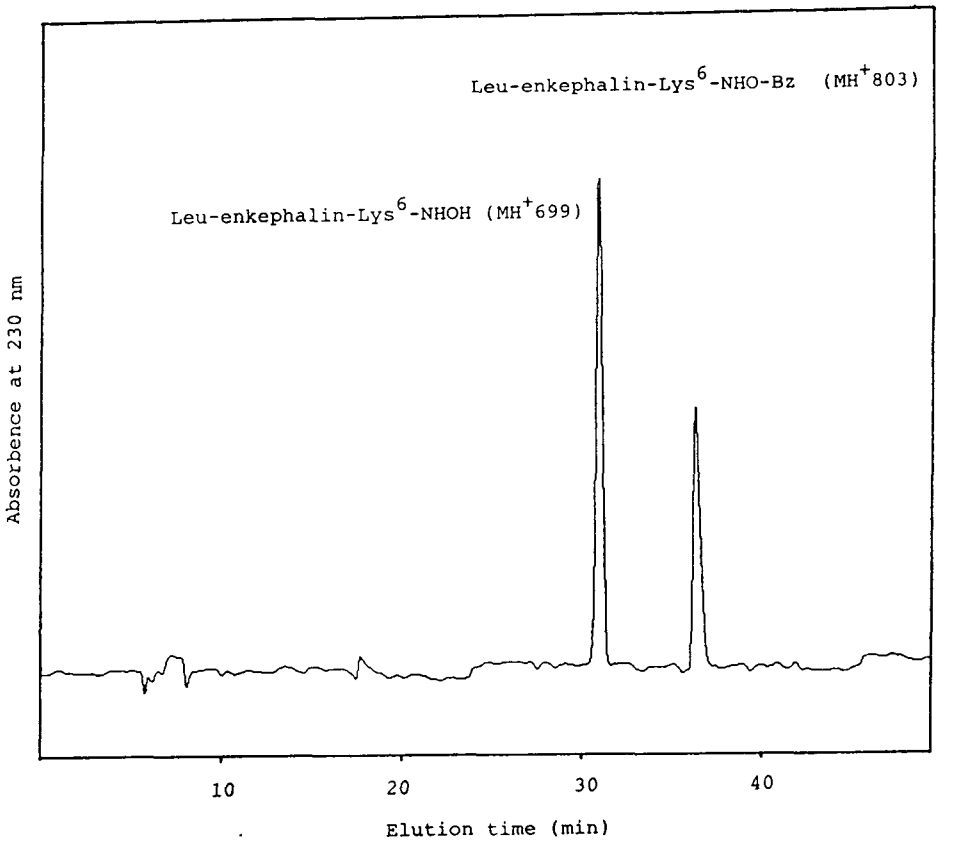


FIGURE 4 Reversed-phase HPLC product analysis. Leu-enk-Lys$^6$-NHO-Bz (37 min elution time) and the product Leu-enk-Lys$^6$-NHOH (31 min elution time) after 40 min incubation of the inhibitor at 37°C in 50 mM Tris-HCl buffer, pH 7.5. Gradient: acetonitrile/water 15–45% (45 min).

(iii) The reaction mechanism of the dynorphin-converting enzyme is completely different from the catalytic mechanism of the "classical" serine proteases.

Recently, E. Shaw and his group have studied the effects of different types of affinity labels on neuropeptide processing enzymes exhibiting similar substrate specificities as hCSF DCE[22–24]. Peptidyl halomethyl ketones, -diazomethyl ketones and -dimethyl sulphonium ketones are known to be effective inactivators of serine and cysteine proteases[25]. Surprisingly, these inhibitors, known to form covalent adducts either with the active site nucleophile (in cysteine proteases) or the acid-base catalyst (histidine, in serine proteases), also fail to irreversibly inactivate the enzymes they were designed for.

From our results and these findings, we speculate that the enzymes highly specific for cationic aminoacid pairs (such as Arg-Arg, Arg-Lys, Lys-Lys, Lys-Arg) might hydrolyze the peptide bonds by a mechanism of substrate-assisted catalysis. The catalytic apparatus of "classical" pancreatic proteases consists of the following catalytic elements:

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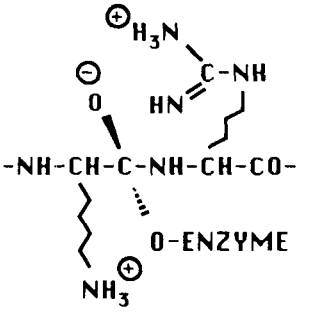
(1) binding: noncovalent interactions occurring between elements of the protein and the substrate, promoting more or less the productive transition to a reaction intermediate, (ii) nucleophilicity of the attacking residue (serine or cysteine), (iii) catalysis of the nucleophilic attack by enzyme groups (tetrahedral intermediate-formation and breakdown to the acyl enzyme), i.e. proton abstraction from the nucleophile and its transfer to the leaving group by the catalytic triad), (iv) transition-state stabilization by the protein matrix (electrostatic stabilization of the oxyanion), and (v) catalysis of the nucleophilic attack of a water molecule at the acyl enzyme (formation and breakdown of a second tetrahedral intermediate)

While stabilization of the tetrahedral intermediates is performed by proton bridging in the so called "oxyanion hole" by protein backbone -NH- groups[26], leaving group departure is acid catalyzed by proton transfer from the active site histidine to the amide[27]. Both tasks could be performed easily by the cationic side chains of the substrates during interaction of neuropeptide processing serine and cysteine enzymes, providing the nucleophile for covalent catalysis and stabilizing the optimal arrangement of the transition state (Scheme II).

Any substrate or inhibitor structure lacking these catalytic features should not interact productively with the enzymes at all. Recent studies on engineered subtilisin, where selectively the involvement of the above factors in the catalysis have been investigated[26], demonstrate that even in the absence of important elements of the catalytic machinery, such as the nucleophile (serine) or the acid-base catalyst (histidine), transition state stabilization by the "oxyanion hole" alone still catalyzes hydrolysis of substrates to a remarkable extent. During reaction of a mutant enzyme lacking the acid-base catalyst (histidine) with a substrate containing a histidine residue proximal to the scissile peptide bond, increased catalysis during interaction of enzyme and substrate is observable[27]. Perhaps, evolution has provided the regulatory peptide/processing enzyme system with a similar feature of substrate-assisted catalysis to add exceptional high specificity and selectivity to the regulatory process.

Future research is directed towards analyzing the mechanistic properties of CSF DCE. The tyrosine-containing inhibitors will be subjected to iodination with $^{125}I$ to be used in further biological studies. The action of arginine- and lysine-containing substrate-analogue diacyl hydroxylamines on processing enzymes such as the coagulation enzymes and the kininases exhibiting trypsin-like specificity will also be studied.




SCHEME 2 Hypothetical transition state during enzymic hydrolysis of the peptide linkage between a cationic amino acid pair.

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## *Acknowledgement*

The work was supported by the Swedish Medical Research Council (Grant No.: 04 X – 3766) and the Swedish National Board for Technical Development.

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