

Lipohexin, a New Inhibitor of Prolyl Endopeptidase from *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096)

II. Inhibitory Activities and Specificity

CLAUDIA CHRISTNER, MARION ZERLIN[†], UDO GRÄFE^{††}, STEPHAN HEINZE^{††},

GERHARD KÜLLERTZ and GUNTER FISCHER^{*}

Max-Planck-Arbeitsgruppe "Enzymologie der Peptidbindung"

Kurt-Mothes-Straße 3, D-06120 Halle/Saale, Germany

[†]Max-Planck-Arbeitsgruppe "Molekulare Zellbiologie"

Drackendorfer Straße 1, D-07747 Jena, Germany

^{††}Hans-Knöll-Institut für Naturstoff-Forschung e.V.

Beutenbergstraße 11; D-07745 Jena, Germany

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The new proline-containing lipohexapeptide lipohexin (**I**) isolated from three fungal strains, *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055 and HKI-0096) is a competitive inhibitor of prolyl endopeptidase (PEP) from human placenta with IC_{50} of 3.5 μ M. Specificity of lipohexin (**I**) is indicated by the much weaker inhibitory activity against bacterial prolyl endopeptidase from *Flavobacterium meningosepticum* (IC_{50} 25 μ M). No effect of lipohexin (**I**) was found on the activity of mechanistically related proteases such as proline specific proteases and other serine proteases.

The prolyl endopeptidase family (PEP) [EC 3.4.21.26] of serine proteases¹⁾, degrades proline-containing peptides by hydrolysis of the peptide bond at the carboxyl side of proline residues. Proteins with molecular weights in the range of 60 ~ 120 kDa showing this enzyme activity were isolated from various microorganisms, plants and invertebrates. In animal tissues, the highest activities were detected in the CNS²⁾. In contrast to aminopeptidases, PEP possesses a high degree of substrate specificity, which is caused by the stereospecific requirements of the amino acid proline³⁾. *In vivo* as well as *in vitro* investigations indicate that PEP plays an important role in the processing and degradation of prolyl-containing biologically active peptides such as angiotensin I and II, oxytocin, vasopressin, neurotensin or angiotensin⁴⁾. Clinical studies suggest PEP involved in the *in vivo* regulation of essential biological functions, e.g. inflammation, homeostasis as well as learning and memory processes³⁾. Furthermore, there is striking evidence that region-specific alteration in the enzyme activity seems to be associated with the pathophysiology of certain infections and diseases like HIV, malaria and several tumors⁵⁾. Abnormal PEP levels may be related to neuropathological disorders, for instance major depression, mania, schizophrenia⁶⁾ and senile dementia of the ALZHEIMER's type⁷⁾.

As specific inhibitors of PEP are expected to have anti-amnesic effects⁸⁾, the chemical synthesis of inhibitors as

well as the search for natural products as possible anti-amnesic drugs is of interest. Recently we isolated the new lipopeptide antibiotic lipohexin (**I**, Fig. 1)⁹⁾. In the preliminary PEP assay lipohexin (**I**) was shown to have a significant inhibitory effect. In this paper the inhibition kinetics as well as the specificity of lipohexin (**I**) towards 18 members of 8 different protease classes will be reported.

Materials and Methods

General Experimental Procedures

Enzyme activities of the different proteases were measured with different methods and devices: Spectrophotometric investigations were performed with an Uvicon 940 (Kontron), a Specord M 500 (Zeiss), or a microplate reader (MR7000, Dynatech). Capillary electrophoresis was carried out with 270A-HT capillary electrophoresis system (Applied Biosystems) and FPLC with L-6210 (Hitachi), column superformance 300-10, BioGPC-Diol 250, 5 μ m (Merck). Analytical HPLC was performed using the column Spherisorb RP18, 4.3 \times 250 mm, 5 μ m.

Chemicals

Chemicals employed were as follows: *N*-succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide (Suc-Ala-Phe-Pro-Phe-*p*NA), Suc-Ala-Ala-Pro-Arg-*p*NA, Suc-Ala-Pro-*p*NA,

Fig. 1. Chemical structure of lipohexin (I).

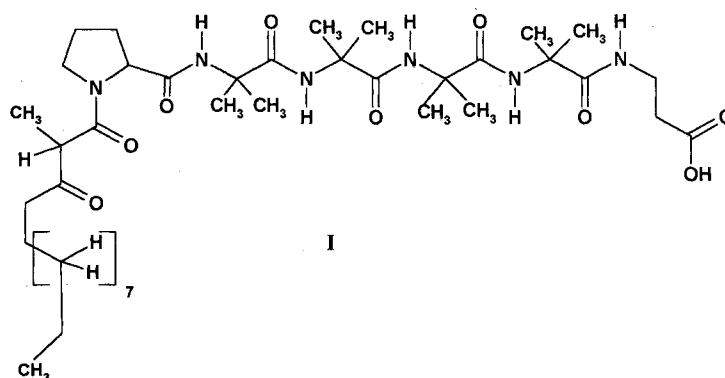


Table 1. Proteinases and proteases tested for the specificity of lipohexin (I).

Enzyme class	Enzyme (EC number)	Source
Aminopeptidases	Prolyliminopeptidase (3.4.11.5) ^{3,13)}	<i>Bacillus coagulans</i>
Dipeptidases	Prolidase (3.4.13.9) ^{3,13)}	Pig kidney
Dipeptidyl peptidases	Dipeptidyl peptidase II (3.4.14.2) ^{3,10)}	Pig kidney
	Dipeptidyl peptidase IV (3.4.14.5) ^{3,10)}	Pig kidney
Metallo carboxypeptidases	Carboxypeptidase A (3.4.17.1) ¹⁴⁾	Bovine pancreas
Omega peptidases	Pyroglutamate-aminopeptidase (3.4.19.3) ¹³⁾	<i>Bacillus amyloliquefaciens</i>
Serine proteases	α -Chymotrypsin (3.4.21.1) ¹⁴⁾	Bovine serum
	Nargarse (3.4.21.14) ¹³⁾	<i>Bacillus amyloliquefaciens</i>
	Pronase (3.4.21.14) ¹³⁾	<i>Streptomyces griseus</i>
	Proteinase K (3.4.21.14) ¹³⁾	<i>Tritirachium album</i>
	Subtilisin (3.4.21.14) ¹³⁾	<i>Bacillus licheniformis</i>
	Thermitase (3.4.21.14) ¹³⁾	<i>Thermoactinomyces vulgaris</i>
	Trypsin (3.4.21.4) ¹⁴⁾	Bovine pancreas
	Thrombin (3.4.21.5) ¹⁴⁾	Bovine plasma
	Prolyl endopeptidase (3.4.21.26) ¹⁰⁾	Human placenta
	Prolyl endopeptidase (3.4.21.26) ¹¹⁾	<i>Flavobacterium meningosepticum</i>
Cysteine proteinases	Papain (3.4.22.2) ¹³⁾	<i>Carica papaya</i>
Aspartic proteinases	HIV-protease (3.4.23.-) ¹²⁾	HIV

Gly-Pro-pNA, Pro-pNA, Ala-Pro-pNA, and Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH₂ from Bachem, Bubendorf, Switzerland; Hippuryl-Phe, Pyroglutamic acid-pNA from Sigma, Deisenhofen, Germany. All other chemicals were of analytical grade.

Lipohexin

The purity of lipohexin (I), isolated from the culture broth of *Moeszia lindtneri* (HKI-0054)⁹⁾ was controlled by analytical HPLC. For inhibitor studies I was dissolved in methanol in a stock solution of 1.3 mM and diluted to concentrations between 100 and 0.05 μ M in the reaction mixture. Inhibition experiments were performed after preincubation of lipohexin (I) with the corresponding enzyme, for at least 10 minutes.

Enzymes

PEP [EC 3.4.21.26] was prepared from human

placenta¹⁰⁾ as a trimeric complex of PEP, α_1 -antitrypsin and human albumin. This trimeric complex, which is not separable under native conditions, was used for the inhibitor studies of lipohexin (I). PEP from *Flavobacterium meningosepticum*¹¹⁾ was obtained from the Weissheimer Research Laboratory, Andernach. Dipeptidyl peptidases II and IV were purified from pig kidney, as previously described¹⁰⁾. HIV-protease was prepared according to the method by Ido *et al.*¹²⁾. The following enzymes were purchased commercially: trypsin (Boehringer, Mannheim, Germany), pronase (Ferak, Berlin, Germany), carboxypeptidase A (Reanal, Budapest, Hungary), papain and thrombin (Serva, Heidelberg, Germany), prolidase, nargarse, subtilisin, thermitase and pyroglutamate aminopeptidase (Sigma, Deisenhofen, Germany), α -chymotrypsin and proteinase K (Merck, Darmstadt, Germany). Characteristics of the used enzymes were compiled in Table 1.

Prolyl Endopeptidase Assay

PEP activity was determined by the cleavage of the chromogenic substrate Suc-Ala-Pro-*p*NA. The increase of absorbance of 4-nitroaniline was measured at 390 nm using the microplate reader. The assay was performed at 30°C. The reaction solution contained enzyme (5.3 nM PEP from human placenta or 51 nM PEP from *Flavobacterium meningosepticum* with specific activities of 9.8 and 3.3 U/ml, respectively), 233 μ M substrate, 100~0.05 μ M inhibitor, 10 mM HEPES, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 150 mM KCl and 0.5 mM DTT, pH 7.8. PEP activity was calculated using an extinction coefficient (ϵ_{390}) of 11500 $\text{M}^{-1}\text{cm}^{-1}$ for 4-nitroaniline^{10). The kinetic constants V_{max} , K_m , and K_i were determined applying the Lineweaver-Burk transformation.}

Assays for Other Enzymes

HIV protease activity was measured spectrophotometrically at 300 nm and 30°C with the Specord M500, using Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH₂ as substrate. The reaction mixture containing 23 μ M HIV protease, 75~20 μ M lipohexin, 100 mM sodium acetate, 1 M NaCl, 5 mM mercaptoethanol, 4 mM EDTA, pH 4.7 was preincubated for 20 minutes at 30°C and the reaction was started by addition of 0.28 mM substrate.

The carboxypeptidase A assay was performed at 228 nm and 37°C in a reaction mixture composed of 2.42 μ M Hippuryl-Phe, 11.3 μ M caboxypeptidase, 75~20 μ M lipohexin, 50 mM Tris-HCl, 1 mM NaCl, pH 7.5.

The activity of all other enzymes was determined analogous to the PEP assay by measuring the concentration of 4-nitroaniline according to the literature cited in Table 1. In the prolidase assay the release of 4-nitroaniline from the substrate cleavage product Pro-4-nitroanilide was reached by the addition of 0.15 μ M prolyliminopeptidase to the reaction mixture^{3). In general, the reactions were started by addition of 25 μ M of 1 mM substrate stock solution. Substrates used are given in Table 2.}

Gel permeation

100 μ l of a mixture consisting of 8 μ g PEP and 0.4 mg lipohexin were applied to a gel filtration experiment. This lipohexin (**I**) concentration was sufficient to inhibit PEP completely. Separation of PEP from lipohexin (**I**) was accomplished by a GPC-Diol column (250, 5 μ m, MERCK) eluted with a buffer solution consisting of 10 mM HEPES, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 150 mM KCl and 0.5 mM DTT (pH 7.8) with a flow rate of 0.5 ml/minute. The enzyme activity was measured by the PEP assay,

described above, with 20 μ l of the protein-containing fractions and 0.37 mM substrate in 100 ml buffer. Analogous, lipohexin (**I**) was detected by measuring the inhibition in a reaction mixture of 5.3 nM PEP, 20 μ l of the FPLC fractions, and 0.37 mM substrate in 100 ml buffer.

Capillary Electrophoresis

Capillary electrophoresis to prove the substrate character of lipohexin (**I**) was performed in 100 mM citrate-NaOH buffer (pH 2.4). A mixture consisting of 30 μ M lipohexin (**I**) and 86 μ M PEP in 10 mM HEPES, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 150 mM KCl and 0.5 mM DTT (pH 7.8) was analysed in intervals of 120 minutes over a period of 24 hours.

Results

Because lipohexin (**I**) showed an inhibitory effect on prolyl endopeptidase from human placenta (hPEP) as well as from *Flavobacterium meningosepticum* (bPEP), the following studies were carried out in parallel with both enzymes.

Inhibition Studies

The Lineweaver-Burk plot (Fig. 2A) reveals a competitive inhibition mode of lipohexin (**I**). The inhibitor constants for hPEP and bPEP were graphically determined to 3.6 and 27 μ M (Fig. 2B), indicating the nearly ten-fold weaker inhibitory activity of lipohexin (**I**) on the bacterial prolyl endopeptidase. Using the formula for a competitive inhibitor model the calculated K_i values of $3.8 \pm 0.7 \mu\text{M}$ (hPEP) and $20.0 \pm 2 \mu\text{M}$ (bPEP) confirm the graphically determined results.

Additionally, the reversibility of the lipohexin-PEP interaction was investigated using gel chromatography. A mixture of lipohexin (**I**) and hPEP, without detectable enzymatic activity in the PEP activity test was separated in fractions containing **I** (66~100) and fractions containing hPEP (45~62). Measurements of the hPEP activity after column passage showed that the enzyme could be reactivated to 79% of the activity compared to the positive control (fractions 45~62). Consequently, lipohexin (**I**) seems to be a reversible inhibitor of PEP.

Lipohexin Stability

Considering the chemical structure of lipohexin (**I**)^{9), which includes a *N*-terminally blocked proline residue as well as a potential cleavage site *C*-terminally to proline, the prolyl-containing lipopeptide could embody a substrate for prolylendopeptidases. This hypothesis was}

examined by controlling the enzyme activity in the presence of lipohexin (I) during a reaction time of 24 hours. The initial enzyme activity was adjusted to 30% of the positive control by addition of $7.5 \mu\text{M}$ lipohexin (I). However, a time-dependent increase of the PEP activity, which should be the consequence of a slow cleavage of lipohexin (I), was not observed. In parallel, a possible enzymatic consumption of lipohexin (I) could not be detected using capillary electrophoresis as a sensitive analytic tool. Consequently, the potential cleavage site for PEP of lipohexin (I) is completely inert even if the competitive inhibition may indicate proper binding to a Michaelis complex.

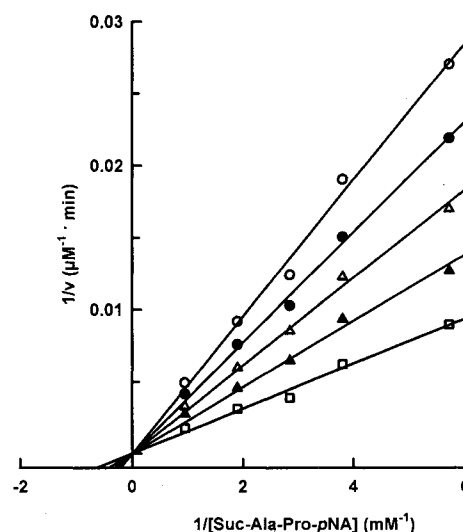
Specificity of Lipohexin Mediated Inhibition

To characterize the specificity of lipohexin (I), representatives of several proteases, which differ with regard to the structure and amino acid sequence of their catalytic centres, were tested in presence of lipohexin (I). Except for an inhibitory effect on the cysteine protease papain ($\text{IC}_{50} = 34 \mu\text{M}$), no further inhibition of the activity of the tested serine proteases, carboxypeptidases, aminopeptidases, dipeptidases, dipeptidyl peptidases, omega peptidases or aspartic proteinases ($\text{IC}_{50} \gg 75 \mu\text{M}$) by lipohexin (I) was detected (Table 2). Interestingly, no inhibitory effects could be observed on the activity of other proline-specific proteases, like HIV-protease, prolidase, prolyliminopeptidase, dipeptidyl peptidase II and dipeptidyl peptidase IV.

Fig. 2. Inhibition kinetics of lipohexin (I) on the activity of prolyl endopeptidase from human placenta.

(A) Lineweaver-Burk plot.

(□) $0 \mu\text{M}$, (▲) $4 \mu\text{M}$, (△) $6 \mu\text{M}$ (●) $7 \mu\text{M}$ and (○) $9 \mu\text{M}$ of lipohexin (I).



(B) Determination of K_i value for lipohexin (I).

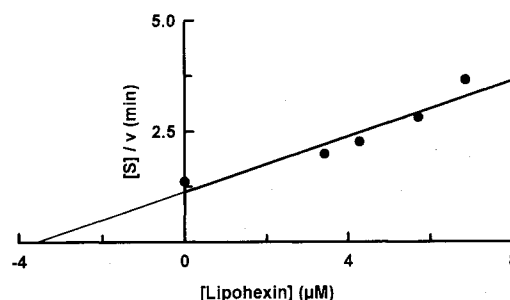


Table 2. Specificity of lipohexin (I) towards members of aminopeptidases, dipeptidases, dipeptidyl peptidases, carboxypeptidases, omega peptidases, serine proteases, cysteine proteinases and aspartic proteases.

Enzyme	Substrate	IC_{50} (μM)
Prolyliminopeptidase	Pro-pNA	$\gg 75$
Prolidase	Suc-Ala-Pro-pNA	$\gg 75$
Dipeptidyl peptidase II	Gly-Pro-pNA	$\gg 75$
Dipeptidyl peptidase IV	Gly-Pro-pNA	$\gg 75$
Carboxypeptidase A	Hippuryl-Phe	$\gg 75$
Pyroglutamate-aminopeptidase	Pyroglutamic acid-pNA	$\gg 75$
α -Chymotrypsin	Suc-Ala-Phe-Pro-Phe-pNA	$\gg 75$
Nargarse	Suc-Ala-Phe-Pro-Phe-pNA	$\gg 75$
Pronase	Suc-Ala-Ala-Pro-Arg-pNA	$\gg 75$
Proteinase K	Suc-Ala-Phe-Pro-Phe-pNA	$\gg 75$
Subtilisin	Suc-Ala-Phe-Pro-Phe-pNA	$\gg 75$
Thermitase	Suc-Ala-Phe-Pro-Phe-pNA	$\gg 75$
Trypsin	Suc-Ala-Ala-Pro-Arg-pNA	$\gg 75$
Thrombin	Suc-Ala-Ala-Pro-Arg-pNA	$\gg 75$
hPEP	Suc-Ala-Pro-pNA	3.5
bPEP	Suc-Ala-Pro-pNA	25.0
Papain	Suc-Ala-Ala-Pro-Arg-pNA	34.0
HIV-protease	Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH ₂	$\gg 75$

Discussion

The majority of the PEP inhibitors described so far are synthetic pyrrolidine derivatives including the especially potent Z-Pro-Prolinal³⁾ and JTP-4819¹⁵⁾. A number of high molecular weight endogenous PEP inhibitors have been purified from different animal tissues³⁾. However, only a few PEP inhibitors from microbial sources have been reported. The linear peptide poststatin¹⁶⁾ from *Streptomyces viridochromogenes* and the acyl-cyclopeptides eurystatins A and B¹⁷⁾ which were isolated from the culture broth of *Streptomyces eurythermus* show potent and specific PEP inhibitory activity.

With lipohexin (I) a new member of the group of microbial PEP inhibitors embodying the essential α -keto- β -amide functionality¹⁷⁾ has been found. In contrast to the previously detected inhibitors, lipohexin (I) represents a combination of a prolyl containing peptide sequence with a long chain fatty acid which may contribute to the characteristic properties of the compound. In general, the fatty acid component of a lipopeptide provides a means of enhancing the lipophilicity and interaction with lipophilic surface structures of peptides. Besides, it may significantly account for the biological stability of the compound¹⁸⁾.

Peptide bonds involving the amino acid proline manifest distinguishing conformational characteristics: the restriction of the ϕ torsion angle to values around 65° and the occurrence of relatively stable *cis* and *trans* conformers¹⁹⁾. As a result these linkages are fairly resistant to the cleavage by broad specificity aminopeptidases. However, a few sets of prolyl-specific peptidases are able to hydrolyze peptide bonds located *N*- or *C*-terminally to proline³⁾. As the prolyl-containing lipohexin (I) contains many features of a substrate or inhibitor, ready to be bound by other proline-specific proteases in addition to bacterial and human PEP, various related peptidases were included in the determination of the specificity of the inhibitory effect.

Surprisingly, lipohexin (I) specifically inhibited PEP whereby an about ten-fold better inhibition of the enzyme isolated from human placenta could be observed, compared to the PEP from *Flavobacterium meningosepticum*. Specificity differences of inhibitors of mammalian and bacterial PEPs were also seen with alkylacyl-prolinal derivatives which strongly inhibited only the bacterial enzyme²⁰⁾. Whether or not the differential inhibition of lipohexin (I) can be influenced by varying the long chain fatty acid will be shown by further investigations.

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