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Natural inhibitors of metalloproteinases from various families of MA clan from wood degrading fungi

Naturalne inhibitory metaloproteinaz różnych rodzin klanu MA z grzybów rozkładających drewno

INTRODUCTION

The activity of proteolytic enzymes, regardless digestive or regulatory ones, is adjusted, despite genetic regulation, by limited proteolysis of precursors (zymogens), by selected degradation of mature enzymes, pH of milieu, cell compartment distribution, and by their natural, endogenous and exogenous, inhibitors. Most of natural protease inhibitors, mainly endogenous, are proteins or peptides, being important factors in controlling proteolysis. It is indicated by their abundance in many cells and tissues, as well as by the variety of their molecular forms characterized from all groups of organisms (3, 4, 12). Some of natural protease inhibitors, mainly exogenous, are also organics (8). Sometimes a change in the level of proteolytic enzyme or/and its inhibitor is connected not only with organism homeostasis, but also with disease or illness. In addition, both metalloproteinases and inhibitors of metalloproteinases have been implicated in several pathological processes including arthritis, tumor growth, tumor metastasis, periodontal illnesses, neuromuscular disease, cerebrovascular disease, and multiple sclerosis (family of matrix metalloproteinase – MMPs from vertebrates) (1,8) and bacterial infection processes (family of thermolysin and family of bacterial collagenase) (13). Fungi in general, and wood degrading (wood rotting) fungi in particular, are very interesting new resources of proteolytic enzymes and their natural inhibitors (5). All catalytic classes of enzymes and some families of natural protease inhibitors were found in them. But the least known are fungal inhibitors of metalloproteinases (8, 9, 14). Therefore the discovery of new, potent inhibitors of metalloproteinases is highly attractive target both scientifically and commercially. This paper describes the extraction and some properties of metalloproteinase inhibitors, both peptides and organics, from fruit bodies and mycelia of wood rotting fungi.

MATERIALS AND METHODS

Materials.

The fruit bodies of wood rotting fungi were collected in forests near Lublin. The mycelia of appropriate species, from Fungal Culture Collection – Lublin, were grown on Fahreus-Reinhammar medium. Extracts were made from fresh fruit bodies and mycelia, or, in some cases, frozen fruit bodies of toadstools or mushrooms.

Extraction of inhibitors of metalloproteinases

Extraction was guided by the gelatinase from *Enterococcus* faecalis inhibition activity of each fraction. Fungi, both fruit-bodies and mycelia, were extracted, by percolation, with appropriate solvent. Extractions were performed with ethanol, methanol, water and 0.1 M Tris-HCl buffer pH 7.0. The recovered filtrate was freeze-dried (7).

Ethanol extracts were concentrated and partitioned between chloroform and water, and then between ethyl acetate and water. Buffer extracts were fractionated by gel filtration chromatography on Sephadex G-50. Preliminary TLC and HPLC analyses, for provisional identification of compounds, were also performed (results not shown).

Determination of metalloproteinase inhibitory activity

Inhibitory activity was measured with the modified method described in (10). General levels of metalloproteinase inhibitor activities were determined by the gelatinase- and keratinase-inhibiting tests according (6), with Z-Pro-Ala-Gly-Pro-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, respectively. Their inhibitory potency was characterized by appropriate metalloproteinase interaction. Inhibition of MMP1, MMP2, MMP3, MMP9, bacterial collagenase and thermolysin were analyzed according (11).

In general, the sample assay: 0.25 ml of a sample was preincubated with the same volume of appropriate metalloproteinase (MMP-1, MMP-2, MMP-3, MMP-9, bacterial collagenase or thermolysin) (8µg/ml) dissolved in 0.1M glycine-HCl buffer (pH 7.5), at 37°C, for 30 min (all enzymes from Sigma, USA). After preincubation, 0.5 ml of 0.1% solution of Azo dye-impregnated collagen (and hemoglobin in the case of thermolysin) (both from Sigma, USA) dissolved in the same buffer was added and the mixture was incubated for 60 min. Then the reaction was stopped by addition of 2 ml of 5% trichloroacetic acid. Samples were centrifuged at 15000g for 10 min, and the absorbance of the supernatant was measured at 520 nm (at 280 nm in the case of thermolysin). The enzyme standard assay: 0.25 ml of a sample was replaced by distilled water. To be sure of inhibition levels, the appropriate TIMP (tissue inhibitors of MMPs) were also used, as cross reference. The described above concentrations of enzymes gave an increase of absorbance at A_{520} of approximately 0.005 OD520 U/min. The percentage of inhibition was calculated as follows: [A_{520} of enzyme standard (+ A_{520} of control) – A_{520} of sample] / [A_{520} of enzyme standard] x 100% (the same calculation at A_{280}).

RESULTS

Novel metalloproteinase inhibitors were preliminary isolated from fruit bodies and mycelia of some wood rotting fungi species. Fungus and mycelium of *Flammulina velutipes* (preliminary screening experiments not shown) were extracted with hexane, ethanol, methanol, water and 0.1 M Tris-HCl buffer pH 7.0 (Table 1). For further experiments extraction with ethanol and buffer was chosen, for all screened and investigated species of wood degrading fungi.

Table 1. Comparison of extraction of inhibitory activities of metalloproteinases from *Flammulina velutipes* fruit-bodies, by various solvents, towards gelatinase from *Enterococcus faecalis*. Inhibitory activity was measured, as described in Materials and Methods

Solvent	Inhibitor activity (% of inhibition)
hexane	20.5
ethanol	74.7
methanol	45.2
water	71.5
buffer	76.9

The first preliminary screening was made for 60 species of wood degrading fungi from nature (fruit bodies) and from laboratory cultures (mycelia) towards gelatinase from *Enterococcus* faecalis (results not shown). The preliminary inhibitory parameters of potency of various type of extracts, activity against gelatinase and collagenase were characterized for six chosen species ((Table 2). In the course of searching for potential pharmacological activities, inhibition of MMP1, MMP2, MMP3 and MMP9 for family M.10, inhibition of bacterial collagenase from Clostridium histolyticum for family M.09, and inhibition of bacterial themolysin from Bacillus thermoproteolyticus for family M.04 of metalloproteinases (2), were analyzed for the best three species – F. velutipes, Cerrena unicolor, and Phlebia radiata (Table 3). Analyses were performed for extracts both from fruit bodies and mycelia. In general, all three fungi are good sources of inhibitors of metalloproteinases, but F. velutipes, especially fruit bodies, seems be the best. Ethanol extracts from mycelium seems better source then ethanol extracts from fruit bodies, and buffer extracts from fruit bodies seems better source then buffer extracts from mycelium (Table 3).

Table 2. Comparison of inhibitory activity of metalloproteinase inhibitors from fruit-bodies and mycelia. The inhibitor samples were incubated with gelatinase from *Enterococcus faecalis* and collagenase from *Clostridium histolyticum*, and inhibitory activity was measured, as described in Materials and Methods

Source of metalloproteinase	Inhibitor activity against gelatinase (% of inhibition)		Inhibitor activity against collagenase (% of inhibition)	
	ethanol	buffer	ethanol	buffer
Bjerkandera adusta				
fruit body	5.5	3.8	0	0
mycelium	8.0	11.3	1.5	2.9
Cerrena unicolor				
fruit body	33.2	62.7	23.6	28.0
mycelium	58.6	78.3	18.8	19.4
Flammulina velutipes				
fruit body	68.6	8.4	22.5	25.2
mycelium	92.5	95.2	29.0	28.8
Phlebia radiata				
ruit body	31.0	59.3	18.0	19.7
mycelium	51.0	66.4	17.2	18.6
Pleurotus ostreatus				
fruit body	19.3	6.8	3.0	5.0
mycelium	11.3	9.7	8.0	7.5
Schizophyllum communae				
fruit body	17.6	9.3	4.5	4.0
mycelium	10.0	12.1	0	0

Table 3. Comparison of inhibitory activity of metalloproteinase inhibitors from fruit bodies and mycelia towards metalloproteinases from clan MA - MMP1, MMP2, MMP3, MMP9, bacterial collagenase and themolysin. The inhibitor samples were incubated with appropriate metalloproteinase, and inhibitory activity was measured, as described in Materials and Methods

Source of metalloproteinase	Inhibitor activity (% of inhibition) against					
	MMP1	MMP2	MMP3	MMP9	collagenase	thermolysin
Ethanol extracts:						
Cerrena unicolor						
fruit body	28.2	36.2	25.7	37.2	23.6	30.7
mycelium	32.4	59.3	30.5	61.5	18.8	33.2
Flammulina velutipes						
fruit body	33.5	72.5	85.4	73.4	22.5	35.6
mycelium	41.8	91.3	86.2	88.6	29.0	37.4
Phlebia radiata						
fruit body	28.4	37.0	55.0	40.5	18.0	28.6
mycelium	35.0	38.2	58.2	38.0	17.2	27.0
Buffer extracts:						
Cerrena unicolor						
fruit body	44.5	64.6	82.4	62.7	28.0	64.1
mycelium	37.9	62.7	75.9	51.5	19.4	58.0
Flammulina velutipes						
fruit body	37.3	18.2	35.0	19.4	25.2	55.9
mycelium	34.0	92.5	40.0	85.3	28.8	59.0
Phlebia radiata						
fruit body	25.0	15.6	28.3	18.2	5.0	43.8
mycelium	32.5	18.2	29.0	20.0	7.5	47.2

Results obtained with TLC and HPLC, after provisional identification, suggest that isolated substances are similar to hydrochinones and polyporenic acids from *Piptoporus betulinus* (in case of ethanol extracts) (8). And low-molecular proteins or high-molecular oligopeptides, like in case of proteinaceous inhibitors of proteases from other catalytic mechanisms (in case of buffer extracts) (2). And they both are very promising bioactive substances (few extracts are really very active).

DISCUSSION

Physiological function of the described substances in organisms of origin, wood degrading fungi, is at the moment unknown. We can only assume that inhibitors active against bacterial metalloproteinses can protect both fruit bodies and mycelia from microbial infections (results against collagenase and thermolysin), and inhibitors active against MMPs can play a role in arrangement in fungal pseudo-tissues (like in animals). This is not surprising because, according to many latest literature data, fungi are quite close related to animals. And for us the most important is inhibitory activity of wood degrading fungi (some of them are medicinal fungi) against MMPs, which is of potential pharmacological application. The natural inhibitory activities directed to metalloproteases, like MMP1, MMP2, MMP3 and MMP9 can be very promising as potential drugs against various diseases and illnesses connected with these enzymes and/or their inhibitors. However, all the drugs considered for treatment of such diseases like a neoplasm, must cross the blood-tissue barrier and the plasma membranes. So both molecular analyses, and *in vivo* and in cell culture experiments should be undertaken.

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SUMMARY

In higher organisms changes in level of proteases and/or their inhibitors are connected not only with organism homeostasis, but also with diseases and illnesses. One of protease clan – MA, contain metalloproteases connected with pathological processes like arthritis, tumor diseases and multiple sclerosis (family of matrix metalloproteinase – MMPs from vertebrates) and bacterial infection processes (family of thermolysin and family of bacterial collagenase). Fungi are very interesting and promising new resources of proteolytic enzymes and their natural inhibitors. All catalytic classes of enzymes and some families of natural protease inhibitors were found in them. But the least known are fungal inhibitors of metalloproteinases From some species of wood rotting fungi, obtained both from fruit bodies from nature and from laboratory, mycelial cultures, new inhibitors of few families from MA clan of metalloproteinases were extracted. Fractions active against metalloproteinases were extracted with few polar and nonpolar solvents. Inhibitory parameters against various families of metalloproteinases were characterized with chosen marker enzymes from concrete metalloproteinase family. Results obtained suggest that isolated substances are both organics (similar to hydrochinones and polyporenic acids from *Piptoporus betulinus*.), and peptides (low-molecular proteins or high-molecular oligopeptides). They have inhibitory activity both against MMPs and bacterial metalloproteinases. And they both are very promising bioactive substances (few extracts are really very active in comparison with available literature data).

Keywords: wood rotting fungi, metalloproteinase inhibitors, matrix metalloproteinases, bacterial metalloproteinases, bioactive substances

STRESZCZENIE

W organizmach wyższych zmiany w poziomie proteaz i/lub ich inhibitorów związane są nie tylko ze stanem homeostazy organizmu, ale także z chorobami i schorzeniami. Jeden z klanów proteaz – MA, czyli metaloproteazy związane z procesami patologicznymi takimi, jak zapalenie stawów, choroby nowotworowe, czy stwardnienie rozsiane (metaloproteazy kręgowców z rodziny metaloproteinaz macierzowych czyli MMPs), czy z procesami infekcji bakteryjnych (rodzina termolizyny i rodzina kolagenaz bakteryjnych). Grzyby stanowią cenne i interesujące źródło inhibitorów proteaz ze

wszystkich typów katalitycznych. Z grzybni kilku gatunków grzybów rozkładających drewno, pozyskanych zarówno z owocników ze stanowisk naturalnych, jak z laboratoryjnej hodowli mycelialnej, wyekstrahowano nowe inhibitory metaloproteinaz z różnych rodzin klanu MA. Frakcje aktywne względem metaloproteinaz ekstrahowano różnymi rozpuszczalnikami polarnymi i niepolarnymi. Parametry inhibitorowe charakteryzowano względem różnych rodzin metaloprotineaz, przy pomocy wybranych enzymów markerowych z konkretnej rodziny metaloproteinaz. Otrzymane wyniki sugerują, że izolowane substancje są zarówno związkami organicznymi (podobnymi do hydrochinonów i kwasów poliporenowych z *Piptoporus betulinus*), jak i peptydami (oligopeptydami lub białkami niskocząsteczkowymi). Wykazują inhibicję zarówno względem MMPs, jaki względem metaloproteinaz bakteryjnych. Substancje te są bardzo obiecującymi związkami bioaktywnymi (niektóre ekstrakty są bardzo aktywne, gdy porówna się je z danymi literaturowymi).

Słowa kluczowe: grzyby rozkładające drewno, metaloproteinazy macierzowe, inhibitory metaloproteinazy, bakteryjne metaloproteinzay macierzowe, substancje bioaktywne