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Natural inhibitors of metalloproteinases from fungi and herbs – new bioactive extracts of pharmacological potential

Naturalne inhibitory metaloproteinaz z grzybów i ziół – nowe bioaktywne ekstrakty o potencjale farmakologicznym

INTRODUCTION

The activity of proteolytic enzymes, regardless of whether they are digestive or regulatory, is regulated by limited proteolysis of precursors (zymogens), by selected degradation of mature enzymes, by pH of milieu, and by their natural inhibitors. Most natural protease inhibitors are proteins or peptides, being important factors in controlling proteolysis, as 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, 11]. Some of them are also organics. Sometimes a change in the level of proteolytic enzyme or its inhibitor is connected with a disease. Fungi are a very interesting new resource 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 less known are fungal inhibitors of metalloproteases (MMPs) [7, 8, 13]. Also, some herbal extracts were found as potential sources of similar substances [2, 6, 12]. In addition, inhibitors of MMPs have been implicated in several pathological processes including arthritis, tumor growth, metastasis, periodontal illness and multiple sclerosis [1, 7]. Therefore, the discovery of new potent inhibitors of MMPs is a highly attractive target both scientifically and commercially. This paper describes the extraction and a few properties of metalloproteinase inhibitors from a few fruit bodies of wood rotting fungi and some herbs.

MATERIAL AND METHODS

M a t e r i a l s . The fruit bodies of wood rotting fungi were collected in forests near Lublin. Extracts were made from fresh or frozen mushrooms at the Department of Biochemistry of UMCS.

Extracts of herbs were prepared both from fresh and dried materials at the Institute of Pharmaceutical Botany, Department of Pharmacy of Medical University of Lublin.

Extraction of inhibitors of metalloproteinases. Extraction was guided by the MMP-1 inhibition activity of each fraction [2]. Fungi and herbs were extracted, by percolation, with chloroform. Some control extractions were performed with hexane, toluene, ethanol and ethyl acetate. The recovered filtrate was freeze-dried. Control, preliminary TLC and HPLC analyses were also performed (results not shown).

Determination of metalloproteinase inhibitory activity. Inhibitory activity was measured with the modified method described in [2, 9]. General levels of metalloproteinase inhibitor activities were determined by the gelatinase- and collagenase-inhibiting tests according [5] to Z-Pro-Ala-Gly-Pro-*p*-nitroanilide and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, respectively. Their inhibitory potency was characterized by appropriate MMPs interactions. Inhibition of MMP1, MMP2, MMP3 and MMP9 was analyzed according [10].

In general, in the sample assay 0.25 ml of a sample was preincubated with the same volume of appropriate metalloproteinase (MMP-1, MMP-2, MMP-3 or MMP-9) (8 μg/ml) dissolved in 0.1 M 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 (Sigma, USA) dissolved in the same buffer was added and the mixture was incubated for 60 min. Then the reaction was stopped by an addition of 2 ml of 5% trichloroacetic acid. Samples were centrifuged at 15000 x g for 10 min, and the absorbance of the supernatant was measured at 520 nm. In the enzyme standard assay 0.25 ml of a sample was replaced by the distilled water. To be sure about inhibition levels, the appropriate TIMP (tissue inhibitors of MMPs) were also used (data not shown). The concentrations of enzymes described above 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%.

RESULTS AND DISCUSSION

Novel metalloprotease inhibitors were preliminary isolated from fruit bodies of a few wood rotting fungi species and some herbs. Fungi and herbs were extracted with chloroform, and some control extractions were performed with hexane, toluene, ethanol and ethyl acetate for comparison (Table 1). Their preliminary inhibitory parameters were characterized (potency of various types of extracts, activity against gelatinases and collagenases) (Table 2). In the course of searching for potential pharmacological activities, inhibition of MMP1, MMP2, MMP3 and MMP9 was analyzed (Table 3). Results (not shown) obtained with TLC and HPLC suggest that isolated substances are similar to berberine (in case of herbs) [12] and polyporenic acids from *Piptoporus betulinus* (in case of fungi) [7]. Organics from our extracts are very promising bioactive substances (a few extracts are really very active).

The physiological function of the described substances in organisms of origin is unknown. Their regulatory role in proteolysis of a fungus or a plant may be considered as inhibitors directed against exogenous metallo-enzymes of pathogens (results against gelatinase and collagenase), or something

quite different. But most important is its inhibitory activity 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 connected with these enzymes. However, all the drugs considered for treatment of such diseases like neoplasm must cross the blood-tissue barrier and the plasma membrane. So *in vivo* or cell culture experiments should be undertaken.

Table 1. Comparison of extraction of inhibitory activities of metalloproteinases towards gelatinase from *Enterococcus faecalis*, from the root of *Eleutherococcus gracilistylus*, with various solvents. Extraction was guided by the MMP-1 inhibition activity of each fraction [2] as in Materials and Methods

Solvent	Inhibitor activity (% of inhibition)
Hexane	20.5
Toluene	64.7
Ethyl acetate	45.2
Chloroform	74.1
Ethanol	25.9

Table 2. Comparison of inhibitory activity of metalloproteinase inhibitors from fungi and herbs. The inhibitor samples were incubated with gelatinase from *Enterococcus faecalis* and collagenase from *Microbacterium* sp., and inhibitory activity was measured, as described in Materials and Methods

Source of metalloiproteinase	Inhibitor activity against gelatinase (% of inhibition)	Inhibitor activity against collagenase (% of inhibition)
Herbs		
Eleutherococcus gracilistylus		
Flos	0	0
Fruit fresh	5.2	0
Fruit dried	2.9	0
Root	74.1	53.2
E. divaricatus		
Flos	0	0
Fruit fresh	3.5	0
Fruit dried	0	0
Root	65.1	100
E. senticosus		
Flos	5.0	0
Fruit fresh	0	9.6
Fruit dried	70.4	33.7
Root	10.8	13,4
Fungi		
Pleurotus ostreatus	50.0	20.0
Bjerkandera adusta	22.0	0
Cerrena unicolor	0	27.8
Auricularia auricula-judae	45.7	15.9
Phlebia radiata	0	10.3

Table 3. Comparison of inhibitory activities of metalloproteinase inhibitors from fungi and herbs towards MMP1, MMP2, MMP3 and MMP9. The inhibitor samples were incubated with appropriate metalloproteinase, and inhibitory activity was measured, as described in Materials and Methods

Source of metalloproteinase	Inhibitor activity against			
	MMP1	MMP2	MMP3	MMP9
	(% of inhibition)			
Herbs				
E.*gracilistylus* Root	74.1	0	62.9	10.5
E. divaricatus Root	39.0	0	52.6	100
E. senticosus Fruit dried	0	0	70.4	0
Fungi				
P. ostr*eatus*	48.5	0	0	21.4
B. adusta	22.9	0	0	11.5
A.auricula-judae	35.7	0	0	5.9

CONCLUSIONS

In this study we described extracts from a few herbs and fungi with inhibitory activities against gelatinases and collagenases. We characterized their preliminary inhibitory parameters against MMP1, MMP2, MMP3 and MMP9, as their potential pharmacological activities. These substances can be very promising as potential drugs against various diseases connected with metalloproteases.

REFERENCES

1. Baker A.H., Edwards D.R., Murphy G.: Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J. Cell Sci., 115, 3719, 2002.
2. Barros I.F.M., Barros P.S.M., Ropke C.D. et al.: Dose dependent *in vitro* inhibition of rabbit corneal matrix metalloproteinases by an extract of *Pothomorphe umbellata* after alkali injury. Brazil. J. Med. Biol. Res., 40, 1129, 2007.
3. Bode W., Huber R.: Natural protein proteinase inhibitors and their interaction with proteinases. Eur. J. Biochem., 204, 433, 1992.
4. Bode W., Huber R.: Structural basis of the endoproteinase-protein inhibitor interaction. Biochim. Biophys. Acta, 1477, 241, 2000.
5. Frederics W.M., Mook O.R.F.: Metabolic mapping of proteinase activity with emphasis on in situ zymography of gelatinases: review and protocols. J. Histochem., 52, 711, 2004.
6. Grimm T., Schafer A., Hogger P.: Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract. Free Radic. Biol. Med., 36, 811, 2004.
7. Kawagishi H., Hamajima K., Inoue Y.: Novel hydroquinone as a matrix metallo-proteinase inhibitor from the mushroom *Piptoporus betulinus*. Biosci. Biotechnol. Biochem., 66, 2748, 2002.

8. Kim W.J., Hong S.C., Do E.J. et al.: *Keumsa linteusan* suppresses invasion of cancer cells through the inhibition of cellular adhesion and MMP-9 expression. Animal Cells Systems, 13, 113, 2009.
9. Lee T-M., Lin Y-H.: Trypsin inhibitor and trypsin-like protease activity in air- or submergence-grown rice (*Oryza sativa* L.) coleoptiles. Plant Sci., 106, 43, 1995.
10. Lombard C., Saulnier J., Wallach J.: Assays of matrix metalloproteinases (MMPs) activities: a review. Biochimie, 87, 265, 2005.
11. Rawlings N.D., Tolle D.P., Barrett A.J.: Evolutionary families of peptidase inhibitors. Biochem. J., 378, 705, 2004.
12. Vayalil P.K., Mittal A., Hara Y. et al.: Green tea polyphenols prevent ultraviolet-induced oxidative damage and matrix metalloproteinase expression in mouse skin. J. Invest. Dermatol., 122,1480, 2004.
13. Weng C.J., Chau C.F., Yen, G.C. et al.: Inhibitory effects of *Ganoderma lucidum* on tumorigenesis and metastasis of human hepatoma cells in cells and animal models. J. Food Agricult. Food Chem., 5049, 2009.

SUMMARY

Novel metalloproteinase inhibitors were extracted from the fruit bodies of a few wood rotting fungi and some herbs. Fractions active against metalloproteases was extracted with chloroform and, for comparison, with other organic solvents. Their inhibitory parameters were characterized against gelatinase and collagenase activities. In the course of searching for potential pharmacological activities, inhibition of MMP1, MMP2, MMP3 and MMP9 were analyzed. The obtained results suggest that isolated substances are similar to berberine (substances from herbs) and polyporenic acids from *Piptoporus betulinus* (substances from wood rotting fungi). They are very promising bioactive substances (few extracts are really very active, in comparison with literature data).

Key words: wood rotting fungi, herbs, metalloproteinase inhibitors, matrix metalloproteinases, MMPs

STRESZCZENIE

Z owocników kilku grzybów rozkładających drewno i kilku preparatów ziołowych wyekstrahowano nowe inhibitory metaloproteinaz. Frakcje aktywne względem metaloproteinaz wyekstrahowano przy użyciu chloroformu, a także dla porównania innymi rozpuszczalnikami. Parametry inhibitorowe scharakteryzowano względem aktywności żelatynaz i kolagenaz. Aby zbadać potencjał farmakologiczny tych aktywności, zanalizowano także inhibicję MMP1, MMP2, MMP3 i MMP9. Otrzymane wyniki sugerują, że izolowane substancje są podobne do berberyny (związki z ziół) i kwasów poliporenowych z *Piptoporus betulinus* (związki z grzybów rozkładających drewno). Substancje te są bardzo obiecującymi związkami bioaktywnymi (niektóre ekstrakty są bardzo aktywne, gdy porówna się je z danymi z literatury).

Słowa kluczowe: grzyby rozkładające drewno, inhibitory metaloproteinaz, zioła, MMPs