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Extraction and Characteristics of Anti-obesity Lipase Inhibitor from *Phellinus linteus*

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To develop a potent anti-obesity lipase inhibitor from mushroom, the lipase inhibitory activities of various mushroom extracts were determined. Methanol extracts from *Phellinus linteus* fruiting body exhibited the highest lipase inhibitory activity (72.8%). The inhibitor was maximally extracted by treatment of a *P. linteus* fruiting body with 80% methanol at 40°C for 24 hr. After partial purification by systematic solvent extraction, the inhibitor was stable in the range of 40~80°C and pH 2.0~9.0. In addition to lipase inhibitory activity, the inhibitor showed 59.4% of superoxide dismutase-like activity and 56.3% of acetylcholinesterase inhibitory activity.

KEYWORDS : Anti-obesity, Lipase inhibitor, *Phellinus linteus*

Various mushrooms have become attractive as functional foods and medicinal agents due to their content of nutraceuticals containing health-stimulating properties and medicinal effects [1]. Many polysaccharides and polysaccharide-protein complexes such as â-glucans and other peptides have been extracted and characterized as bioactive compounds from the fruiting bodies and mycelial of mushrooms [2-8].

Recently, obesity has received considerable attention due to its promotion of cardiovascular disease and cancer. There are several known anti-obesity agents such as amylase inhibitors and pancreatic lipase inhibitors; additionally, there are commercial anti-obesity products such as sibutramine and orlistat (Xenicol) [9]. Especially, lipase (triacylglycerol hydrolase, EC 3.1.1.3) is a key enzyme for dietary fat adsorption, hydrolyzing triacylglycerols to 2-monoacylglycerols and fatty acids. Therefore, it is generally thought that a potent lipase inhibitor could be very useful as an anti-obesity compound.

There have been many reports on lipase inhibitors derived from natural sources such as phytic acid [10], tannin [11], algae [12], pumpkin and Job's tear [13], saponins [14], Rhei Rhizoma and chunghyuldan [15], soybean and oil seeds [16] and *Monascus* pigment [17], etc. It is also well known that bovine serum albumin and β-lactoglobulin contain lipase inhibitors [18]. Recently, orlistat, a potent lipase inhibitor produced by *Streptomyces toxytricini*, has been proven useful for the treatment of obesity [19]. However, commercial lipase inhibitors have some side effects such as fecal incontinence and low efficiency, etc. Furthermore, there have been relatively few studies on the development of anti-obesity lipase inhibitors from natural sources without side effects or lipase inhibitors from edible or medicinal mushrooms.

In this paper, we describe the screening of a potent lipase inhibitor-containing mushroom as well as the optimal extraction conditions of lipase inhibitor from the fruiting body. Furthermore, we describe the properties of the partially purified inhibitor and determine the inhibitor may be an effective bioactive anti-obesity agent.

Materials and Methods

Mushrooms and chemicals. Mushrooms were obtained from Chungnam Agricultural Research and Extension Services (Yesan, Korea) and Baekma Agricultural Community of Buyea in Chungnam province. Unless otherwise specified, all chemicals and solvents were of analytical grade. Lipase (porcine pancreatic lipase, Type II), triolein as substrate, TES (N-Tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid), taurocholic acid, gum arabic, HHL (Hip-His-Leu), pyrogallol, acetylcholinesterase, thrombin and fibrinogen were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of extracts. Fruiting bodies were lyophilized and powdered. Mycelial were inoculated in potato-dextrose broth and cultured at 28°C for 5 days. The seed cultures were inoculated in whole grain and then cultured at 28°C for 15 days. The cultured mycelial were lyophilized, powdered and filtered using a 20 mesh sieve.

The lyophilized powders of fruiting bodies and mycelial were added to methanol (1 : 20, w/v) and shaken for 20 hr at 40°C. These extracts were both centrifuged at

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4,000 ×g for 20 min, concentrated using a rotary vacuum evaporator and then lyophilized.

Assay of lipase inhibitory activity. Lipase inhibitory activity was determined by measuring the rate of release of oleic acid from triolein according to the modified method of Bitou *et al.* [12]. A suspension of 120 mg of triolein, 90 mg of gum arabic, 10.16 mg of taurocholic acid in 9 mL of 0.1 N TES buffer (pH 7.0) containing 0.1 M NaCl was sonicated for 5 min. A mixture of 50 μL of pancreatic lipase (500 U/mL), 50 μL of mushroom extracts (4 mg/mL) and 300 μL of substrate mixture was incubated for 30 min at 37°C, and the amount of oleic acid produced was determined by the method of Zapf *et al.* [20] with slight modification. The 400 μL incubation mixture was added to 3 mL of chloroform/hexane (1 : 1) containing 2% (v/v) ethanol and extracted by shaking for 10 min. The mixture was centrifuged (2,000 ×g) for 10 min followed by the addition of copper reagent to the lower organic layer and shaking for 10 min. The mixture was centrifuged (2,000 ×g, 10 min), and 1 mL of the upper organic layer containing the copper salts of the extracted oleic acid was reacted for 10 min with 0.5 mL of 0.1% (w/v) bathocuproine-chloroform solution containing 0.05% (w/v) 3-tert-butyl-4-hydroxyanisol. The absorbance was determined at 480 nm. The inhibition (%) was calculated using the equation.

$$\text{Inhibitory activity (\%)} = (A - B)/A \times 100,$$

where A is lipase activity in the reaction solution without sample and B is lipase activity in the reaction solution containing sample.

Determination of physiological function. The partially purified lipase inhibitor (inhibitory activity; 60%/mg solid) was dissolved in 50 μL of D.W and physiological functions were determined as follows. We used the Blois method [21] and DPPH to assay antioxidant activity. Superoxide dismutase (SOD) - like activity was assayed by the method of Marklund *et al.* [22].

The inhibition of angiotensin I-converting enzyme (ACE) by the partially purified lipase inhibitor was assayed according to the modified method of Cushman and Cheung [4, 23]. Acetylcholinesterase (AChE) inhibitory activity was measured spectrophotometrically using the technique of Ellman *et al.* [24]. Fibrinolytic activity was also assayed by the method of Fayek and El-Sayed [25].

Results and Discussion

Screening of a potent lipase inhibitor-containing mushroom. To select the most potent lipase inhibitor-containing mushroom, methanol extracts from 14 species of mushroom fruiting bodies and 108 species of mushroom mycelial were tested for their lipase inhibitory activities. Among the fruiting bodies tested, *P. linteus* (Goryo No. 1) showed the highest lipase inhibitory activity (72.5%). Methanol extracts of *Fomitopsis pinicola* (68.0%) and *Innotus obliquus* (62.9%) also showed high inhibitory activities (Table 1). Generally, methanol extracts from mycelial showed higher lipase inhibitory activities when compared to fruiting bodies. Methanol extract of *P. linteus* (ASI 26082) mycelial showed the highest lipase inhibitory activity (70.5%), although mycelial extracts of *Ganoderma applanatum* ACTC 44053 (67.1%), *Agrocybe aegerita* ASI 19003 (65.3%), *Pleurotus ostreatus* Choogwang (64.7%) and *Ganoderma lucidum* MR15008 (64.7%) also showed high inhibitory activities (Table 2). Since methanol extracts of *P. linteus* (Goryo No. 1) showed the highest lipase inhibitory activity, the *P. linteus* fruiting body was selected for the production of lipase inhibitor. This is the first report on the development of an anti-obesity nutraceutical based on lipase inhibitor from *P. linteus*, even though *P. linteus* has been studied for its immune-stimulating action by polysaccharides and anti-cancer activity [26-29].

Table 1. Lipase inhibitory activities of methanol extracts from mushroom fruiting bodies

Scientific name	Origins	Inhibitory activity (%)
Cordyceps militaris	Yedang No. 3	10.8
Hericium erinaceum	Noru No. 2	13.5
Ganoderma lucidum	Yeongji No. 3	35.7
Inonotus obliquus	Chaga No. 1	62.9
Phellinus linteus	Goryo No. 1	72.5
Ganoderma lucidum	Yeongji No. 1	44.5
Agaricus blazei	Sinryeon No. 1	39.4
Pleurotus osteratus	Miso No. 1	53.4
	Jangan No. 50	36.3
Lentinus edodes	No. 357	45.3
Collybia velutipes	Baekro No. 1	35.4
Auricularia auricula	Moki No. 1	41.0
Agaricus bisporus	Agro No. 103	43.6
Fomitopsis pinicola	Sonamujamnabi No. 1	68.0

Therefore, *P. linteus* (Goryo No. 1) may play a functional role in the food industry. Meanwhile, the lipase inhibitory activity of *P. linteus* was similar or lower than those of several other lipase inhibitors such as *Monascus* pigment derivatives from *Monascus fermentation* [17], soybean and other oil seeds [16], *Rhei Rhizoma* and *Chunghyuldan* [15], *Acanthopanax sessiliflorus* leaves [14] and marine algae [12].

Optimal extraction conditions. Optimal extraction conditions of lipase inhibitor from *P. linteus* fruiting body were found to be in the range of 40~100°C and from 6 hr to 48 hr using methanol. As the methanol concentration and extraction time were increased, lipase inhibitory activity was increased. Lipase inhibitor was maximally extracted

Table 2. Lipase inhibitory activities of methanol extracts from mushroom mycelial

Scientific name	Origins	Inhibitory activity (%)	Scientific name	Origins	Inhibitory activity (%)
Agaricus arvensis	MKACC52925	47.8		KNU 73	45.9
Agaricus augustus	MKACC52915	57.8		MRI 5012	11.4
Agaricus bisporus	MKACC52236	45.4	*Ganoderma mirabile*	CBS 218. 36	51.4
	52241	50.3	*Ganoderma neo-japonicum*	NJ-03	n.d
	52242	50.5		KNU 28	n.d
	52980	40.2	*Ganoderma oerstedii*	ATCC 52411	24.3
Agaricus blazei	MKACC53398	44.4	*Ganoderma oregonense*	MRI 5006	67.4
Agaricus marginela	MKACC52926	45.1		ATCC 64487	22.9
Agaricus placomyces	MKACC52917	48.1		46751	48.2
Agaricus silyicola	MKACC52919	52.5	*Ganoderma pfeifferi*	CBS 747.84	40.4
Agaricus sp.	MKACC52927	48.2	*Ganoderma resinaceum*	ATCC 52416	32.4
	52928	40.3		52413	34.2
	Yangsongee No. 707	44.7	*Ganoderma* sp.	CNRG 13	43.4
Agaricus subrutilescens	MKACC52918	58.0		14	45.3
	52921	47.4		21	35.1
	52922	57.3		25	37.9
	52923	53.7		32	35.6
	52924	63.9		34	41.6
	52911	52.0		KNU 87	42.0
Agrocybe aegerita	ASI 19003	65.3		CNRG 20	47.9
	KCTC16817	52.7	*Ganoderma subamboinense*	ATCC 52420	39.8
	16819	50.2		52419	35.4
	16820	45.3	*Ganoderma tsugae*	ATCC 46754	38.5
	16821	46.1		64795	40.1
	16823	45.4	*Grifola frondosa*	Ip sae No. 1	48.5
Agrocybe alnetorum	KCTC16853	58.4		China No. 1	48.1
Agrocybe firma	KCTC16824	57.1	*Lentinus edodes*	LE1	44.8
Agrocybe paludosa	KCTC16826	54.5		LE2	47.1
	16828	59.0	*Lentinus edodes*	LE3 (choongo)	55.7
	16825	58.3		LE4 (dongo)	54.7
Agrocybe pediades	KCTC16830	54.0		LE5 (135)	51.9
Agrocybe praecox	KCTC16831	52.0		LE6 (939)	52.9
Agrocybe vervacti	KCTC16832	52.6		LE7 (9015)	53.8
Coriolus brevis	ASI 16007	50.4		LE8 (yunam)	46.0
Coriolus versicorlor	ASI 16008	49.1	*Lyopyllum ulmarium*	ASI 8007	48.1
	16001	51.5	*Phellinus linteus*	ASI 26013	58.9
	16003	53.4		26011	67.6
	16004	51.5		26020	68.9
	16005	49.9		26038	67.9
Coriolus versicorlor	ASI 16006	49.9		26056	68.7
Flammulina velutipes	Paengee No. 2	53.3		26081	70.5
Ganoderma applanatum	ATCC 44053	67.1	*Phellinus* sp.	Choongju No. 2	69.2
Ganoderma lucidum	ATCC 64251	66.9	*Pholiota nameko*	ASI 5016	45.9
	46755	62.8	*Pleurotus cornucopiae*	Norang neutaree	44.3
	ASI 7002	63.0	*Pleurotus eryngii*	ASI 2155	52.2
	7003	64.0	*Pleurotus ostreatus*	Nonggi No. 2-1	18.2
	7024	61.0		Soojip No. 1	55.1
	7005	64.2		Choogwang	64.7
	7019	61.3		Dangjin No. 1	15.3
	MRI 5005	57.0		Wonhyung	51.3
	5008	64.7		Nonggi No. 201	8.7
	5002	64.3		202	n.d
	5010	53.1		Aeneutaree No. 2	n.d
	CNRG 33	38.5		Chunchoo No. 1	n.d

n.d, not detected.

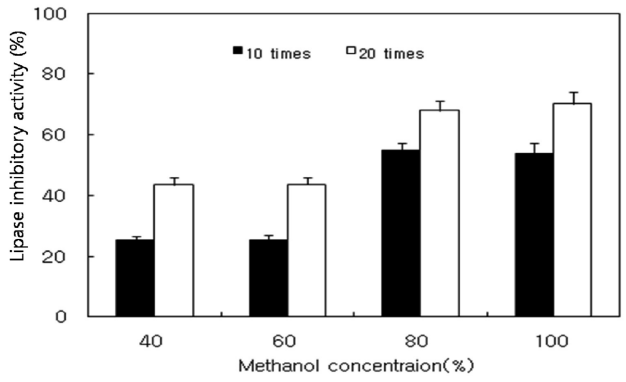

Fig. 1. Effects of methanol concentration on the extraction of lipase inhibitor from *Phellinus linteus*.

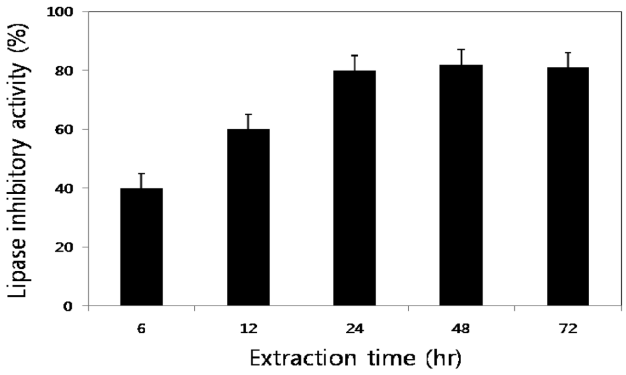

Fig. 2. Effects of extraction time on the extraction of lipase inhibitor from *Phellinus linteus*.

by 80% methanol (1 : 20) for 24 hr at 40°C and then remained constant without any change (Figs 1 and 2). Seo *et al.* [1] reported antidementia β-secretase inhibitor was maximally extracted at 40°C for 24 hr by 50% methanol. Further studies on extraction by water or ethanol are necessary for application of lipase inhibitor from *P. linteus* to the food industry.

Characteristics of the lipase inhibitor. The lipase inhibitor from *P. linteus*, partially purified by systematic solvent extraction, was soluble in water and methanol, with maximal absorption spectra of 225.1 nm (data not shown). Stability was investigated in the temperature range of 40~100°C and pH 2.0~9.0.

Treatment for 60 min at 40°C and 60°C did not affect inhibitory activity, and only 16.2% of the relative inhibitory activity was decreased by treatment at 100°C for 60 min (Fig. 3).

Furthermore, the partially purified lipase inhibitor showed over 80% relative inhibitory activity in the range of pH 2.0~9.0 (Fig. 4). From these results, we conclude that lipase inhibitor from *P. linteus* is very stable under heat (40~80°C) and a wide pH range (2.0~9.0), suggesting it may be very useful in the food or medicinal industries.

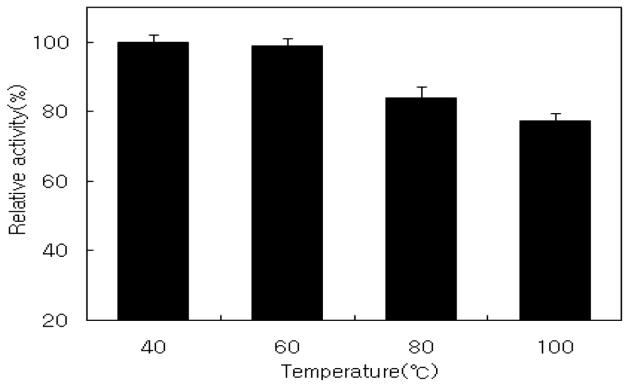

Fig. 3. Thermal stability of the partially purified lipase inhibitor from *Phellinus linteus*. The partially purified lipase inhibitor was treated for 60 min at various temperatures and its relative inhibitory activity was determined.

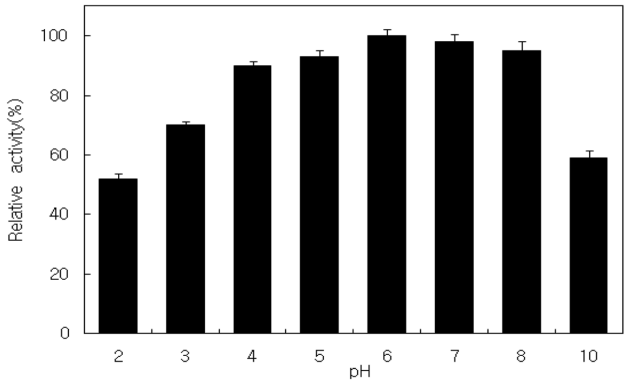

Fig. 4. pH stability of the partially purified lipase inhibitor from *Phellinus linteus*. The partially purified lipase inhibitor was treated for 60 min at various pH and its relative inhibitory activity was determined.

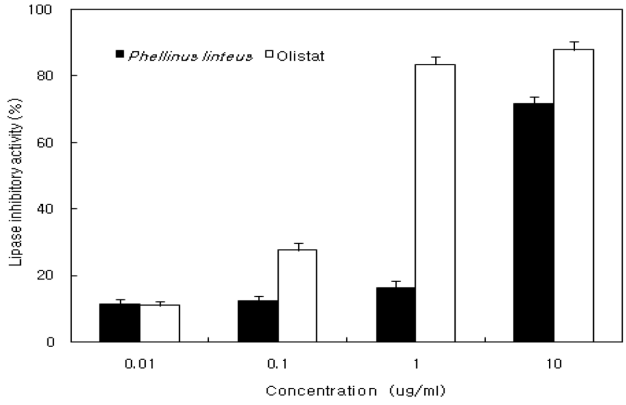

Fig. 5. Comparison of inhibitory activity between lipase inhibitor from *Phellinus linteus* and commercial lipase inhibitor (Olistat).

We compared the activities of lipase inhibitor from *P. linteus* and the commercial lipase inhibitor Olistat at various concentrations (Fig. 5). Olistat showed over 83%

Table 3. Physiological functionalities of methanol extract from *Phellinus linteus*

	Antioxidant activity (%)	ACE inhibitory activity (%)	SOD-like activity (%)	Fibrinolytic activity	AChE inhibitory activity (%)
Methanol extract	11.2 ± 0.6	n.d	59.4 ± 0.7	n.d	56.3 ± 0.5

ACE, angiotensin I-converting enzyme; AChE, acetylcholinesterase.; n.d, not detected.

inhibitory activity at 1.0 μg per mL and 10 μg per mL, whereas the partially purified lipase inhibitor from *P. linteus* showed only 71.5% inhibitory activity at 10.0 μg per mL, lower than that of commercial Olistat.

Some physiological functions of the partially purified lipase inhibitor were determined (Table 3). In addition to lipase inhibitory activity, the inhibitor showed 59.4% SOD-like activity and 56.3% anti-dementia acetylcholinesterase inhibitory activity. However, antioxidant activity was 11.2%, and antihypertensive ACE inhibitory activity and fibrinolytic activity of *P. lenteus* were not detected, even though some ACE inhibitors are present in several mushrooms including *Pholiota adiposa* [4].

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