クした痛みが生じ、筋肉痛、呼吸困難、テタニー、不整脈が起こるとされる。本事例において、スギヒラタケ摂取によると見られる重篤な痙攣重積（テタニー）という臨床所見があることは、候補化合物はビタミンDアンタゴニストである可能性があり、この候補加工物との脳症との関連性が示唆された。

<u>両腎摘出ラットにおけるシアンカリウム投与の検討</u>

E. 結論

2005年度から2006年度にかけてスギヒラタケ摂取と関連したと思われる急性脳症の症例報告がされている[2)-5)]。それら報告に共通して書かれている症状は、振戦、脱力、構語障害、意識障害、全身ケイレン、脳画像所見（MRI）では、両側基底核病変　レンズ核（被核と淡蒼球）で病変が見られ、脳波では山形県で２例でCreutzfeldt-Jacob病と類似するPSD（周期性同期性放電(periodic synchronous discharge; PSD)）が認められている。

これら論文においてメタノール中毒、マンガン中毒、3-ニトロプロピオン酸中毒（サトウキビカビ毒）、青酸（シアン）中毒と類似の脳症であることが示唆されている。しかしながら、メタノール中毒では視力障害はみとめられるが、これら報告ではみとめられていないこと、またマンガン中毒では淡蒼球病変が主体で、昨年度の報告から多量に検出されていないことから、メタノール中毒やマンガン中毒とは考えにくい。従って、3-ニトロプロピオン酸中毒(サトウキビカビ毒）か青酸（シアン）中毒が考えられるが、現在のところUV-HPLC分析では3-ニトロプロピオン酸が検出されていないことから、3-ニトロプロピオン酸中毒（サトウキビカビ毒）とも考えにくい。

以上のことと本研究によりシアンイオンが比較的高値で検出されていたことを考えると、シアン中毒の可能性が考えられる。シアン中毒は高酸素需要性組織に強い親和性をもち、出血性壊死を誘引する。特に大脳基底核また大脳皮質、感覚運動野皮質にも影響するとの報告があることから[6)]、シアン中毒は今回の症例報告に近い病変であることが考えられる。

1940年頃から1980年頃にかけての論文報告において、シアン中毒と脳症との関連に関する論文が多数報告されている[7)-11)]。また、シアン投与の自殺者には大脳基底核病変がおこることがあると報告されている[12)-13)]。さらに喫煙者の血清中のチオシアン酸イオンと脳梗塞との関連や尿毒症患者における喫煙によるシアンイオン、チオシアン酸イオンの蓄積があることが報告されている[14)-15)]。

一方、チオシアン酸イオン（SCN-）は脳内グルタミンレセプター（AMPA）receptorとグルタミン酸とのアフィニティーを数倍上昇させる働きがあり[16)-20)]、AMPA receptorとグルタミン酸との結合が過剰反応になると振戦、脱力、歩行困難、立ち上がり困難、痙攣性麻痺がおこることが示唆されている。

以上のことから、腎障害患者がシアンイオンを大量に摂取した場合、ロダニースによりチオシアン酸イオンの生成が予想されるが、尿からの排泄が困難になり、体内に蓄積される可能性がある。蓄積されたチオシアン酸イオンは、今

回のスギヒラタケ摂取と同様な症状がおこる可能性がある。しかしながら他の成分による可能性もあるため今後も検討が必要と思われる。

またメタボローム TOF-MS 手法を用いた解析から、「スギヒラタケ群と市販きのこ群（シイタケ、マイタケおよびシメジ）」とが群で分けられ、さらにスギヒラタケ群は「脳症報告例なし」、「数件の報告例がある」、「（死者を含む）多数の報告例がある」の３群に分類できた。「多数の報告例がある」地域群のスギヒラタケで共通して検出され、「脳症報告なし」地域群で検出されないピークを抽出したところ、３成分のプロビタミン D 類似関連化合物が考えられた。このプロビタミン D 類似関連化合物と急性脳症との関連も今後検討すべき課題である。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の登録

なし

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表 1. シアンイオン及びチオシアン酸イオン分析結果（2004 年度産スギヒラタケ）

Sample No.	Producing district	CN^- (μg / g dry weight)	SCN^- (μg / g dry weight)
Sample 1	Akita 1	12.73	1.13
Sample 2	Akita 2	25.49	4.59
Sample 3	Yamagata 1	1.01	1.07
Sample 4	Yamagata 2	7.20	0.87
Sample 5	Niigata 1	1.84	0.48
Sample 6	Niigata 2	56.17	17.03
Sample 7	Mie	3.06	1.64
Sample 8	Gifu 1	22.10	10.34
Sample 9	Gifu 2	113.95	9.37
Sample 10	Fukui 1	3.00	1.09
Sample 11	Fukui 2	0.86	1.41
Sample 12	Ishikwa	N. D.	0.10
Sample 13	Kyoto	1.23	0.16
Sample 14	Ibaraki	96.63	8.38
Sample 15	Fukushima 1	0.58	0.08
Sample 16	Fukushima 2*	0.29	N. D.

＊2003年度産，缶詰

表２．シアンイオン及びチオシアン酸イオン分析結果（2004 年度産スギヒラタケ）

Sample No.	Producing district	CN^- (μg / g dry weight)	SCN^- (μg / g dry weight)
Sample 1	Iwate 1	7.52	2.40
Sample 2	Iwate 2	3.29	0.65
Sample 3	Yamagata 1	1.54	1.10
Sample 4	Yamagata 2	12.12	1.32
Sample 5	Miyagi 1	1.16	1.91
Sample 6	Miyagi 2	30.98	3.43
Sample 7	Miyagi 3	3.89	0.91
Sample 8	Shimane	0.90	2.37
Sample 9	Nagano 1	1.38	0.24
Sample 10	Nagano 2	7.00	4.51
Sample 11	Aichi	3.83	3.29
Sample 12	Fukui	5.71	0.75
Sample 13	Tokyo	2.60	0.75
Sample 14	Akita	6.78	2.17
Sample 15	Fukushima 1	3.65	2.05
Sample 16	Fukushima 2	2.68	0.45
Sample 17	Hyogo 1	1.80	0.18
Sample 18	Hyogo 2	9.57	2.42
Sample 19	Wakayama	2.01	0.30
Sample 20	Shiga	2.81	1.91
Sample 21	Ishikawa	19.23	6.41
Sample 22	Niigata	3.71	1.06
Sample 23	Gifu	3.94	0.30

表 3.　食用キノコ中のシアンイオン・チオシアン酸イオン濃度の定量

		CN^- (μg / g dry weight)	SCN^- (μg / g dry weight)
Mushroom	*Agaricus bisporous*	0.20	0.26
Shiitake	*Lentinus edodes*	0.20	0.88
Agaricus	*Agaricus Blasei Murrill*	N.D.	0.52
Bunashimeji	*Hypsizigus marmoreus*	0.19	0.20
Hiratake	*Pleurotus ostreatus*	0.66	3.87

表４．β-グルコシダーゼ，β-ガラクトシダーゼ，β-マンノシダーゼ消化による検討

Sample No.	Producing district	CN^- (μg / g dry weight)	SCN^- (μg / g dry weight)
Sample 1	Akita Blank	5.2	3.8
Sample 2	Akita Glcosidase	6.9	5.2
Sample 3	Akita Galactosidase	6.2	6.5
Sample 4	Akita Mannosidase	5.1	2.3
Sample 5	Gifu Blank	125.9	14.0
Sample 6	Gifu Glcosidase	129.8	6.5
Sample 7	Gifu Galactosidase	96.9	6.3
Sample 8	Gifu Mannosidase	115.8	6.5
Sample 9	Ibaraki Blank	75.4	8.3
Sample 10	Ibaraki Glcosidase	80.9	10.6
Sample 11	Ibaraki Galactosidase	90.2	16.6
Sample 12	Ibaraki Mannosidase	79.1	14.9

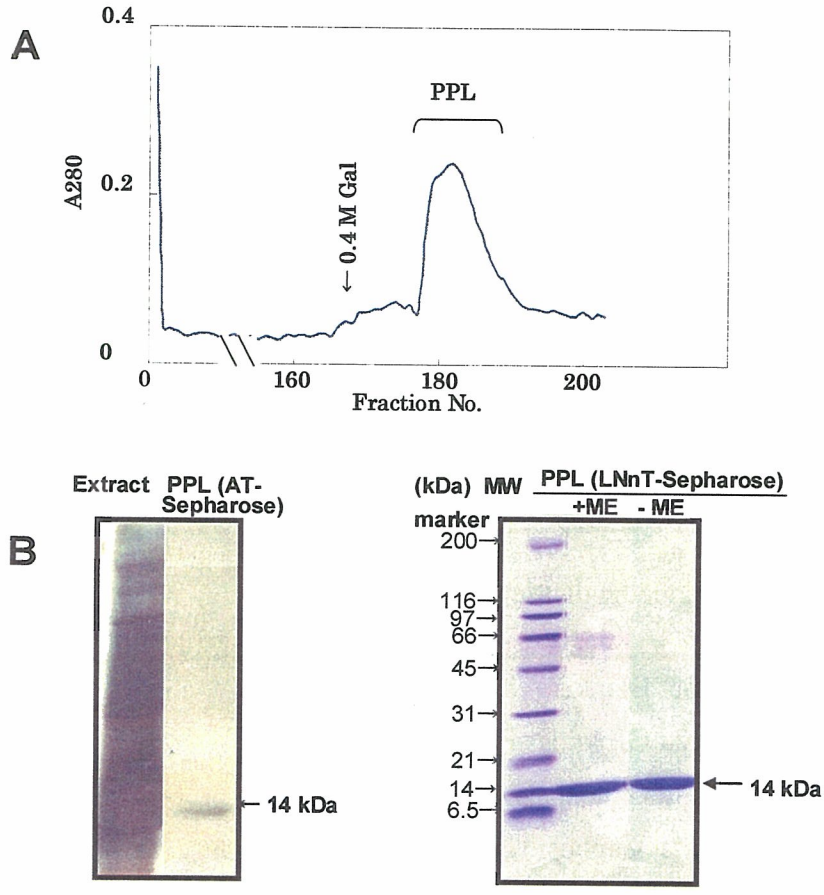


Fig. 1　　*Pleurocybella porrigens* レクチン（PPL）の精製

(A)　酸処理Sepharoseカラムを用いたPPLのアフィニティークロマトグラフィー

(B)　精製PPLのSDS-PAGE　13.5 % ポリアクリルアミドゲル．還元下、酸処理Sepharoseカラムからの溶出画分（左図）．還元下・非還元条件下でのLNnT-Sepharoseカラムからの溶出画分（右図）．

Table 5　PPLのアミノ酸組成

アミノ酸	PPL	
	LNnT-Sepharose	酸処理-Sepharose
	(mol %)	
Asx	11	10
Thr	11	11
Ser	7.0	7.3
Glx	7.0	8.3
Pro	5.7	3.7
Gly	14	14
Ala	10	11
Cys*	0	0
Val	7.0	6.6
Met	0.0	<1.2
Ile	6.4	5.9
Leu	8.3	8.7
Tyr	1.9	1.7
Phe	1.9	1.6
Lys	0.6	10
His	1.9	1.4
Arg	5.1	4.4
Trp**	-	2.8

*Cys　ピリジルエチル化後、水解して分析.

**Trp　メタンスルホン酸中、加水分解して分析.

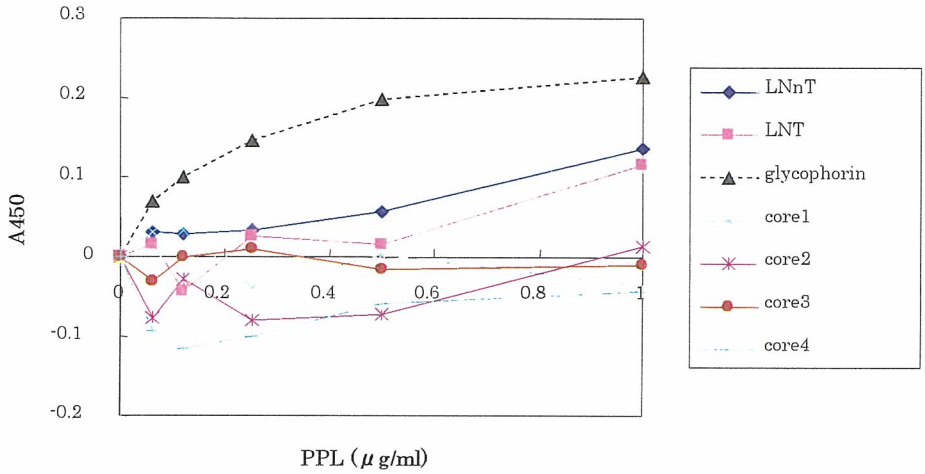


Fig. 2 PPL と 糖 BP-プローブとのELISAによる反応性試験

Table 6 PPL と糖 BP-プローブとのELISAによる反応性

Biotin hydrazide-glycophorin glycan	+++	BP-Lac	+
BP-LacNAc	+++	BP-α-, β-Gal	-
BP-α-Fuc	++	BP-α-Man	-
BP-β-Glc	++	BP-α-Man6-P	-
BP-LNnT	++	BP-β-GlcNAc	-
BP-LNT	++	BP-α-, β-GalNAc	-
BP-α-Glc	+	BP-α-Neu5Ac	-
BP-$HSO_3$3'-core 1	++	BP-core 1, 2, 3, & 4	-
		BP-sialyl Lex	-

-: 非結合, +: 低結合, ++: 結合, +++: 高結合

LNnT: lactoN-neotetraose (Galβ1,4GlcNAcβ1,3Galβ1,4Glc), LNT: lactoN-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc) , core 1: Galβ1,3GalNAc

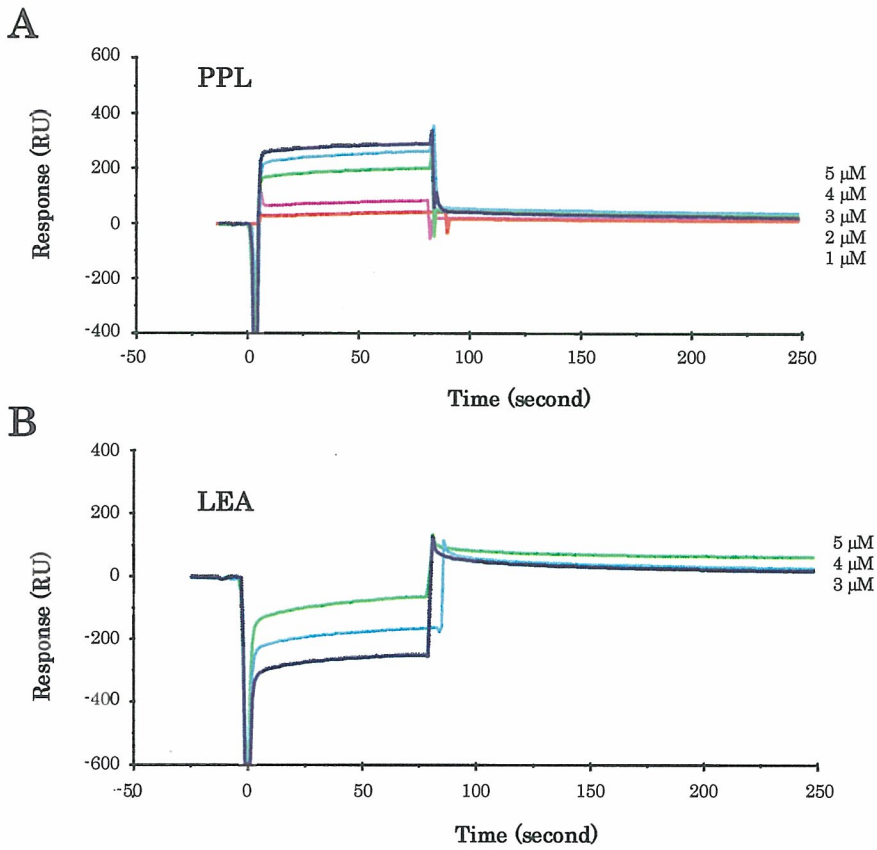
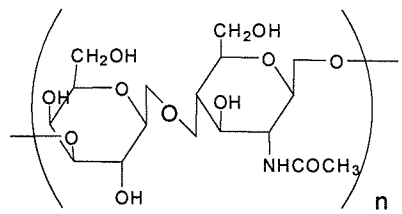


Fig. 3 SPRによるPPLおよびLEAとウシグリコホリンとの結合性解析
(A) PPL-固定化フローセル (B) LEA-固定化フローセル.
緩衝液: 10 mM HEPES-0.14 M NaCl
流速: 20 μl/min 温度: 25℃.
固定化量 (RU): BSA 461, PPL 9327, LEA 10934.
グリコホリン濃度: 分子量を250 kDaとして計算し、図中に表示

Table 7 PPL およびLEAとウシグリコホリンとの結合パラメーター

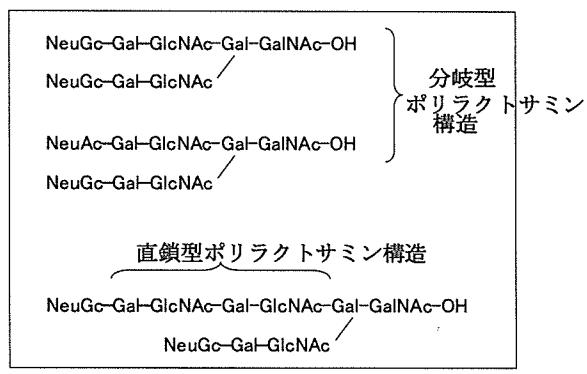
	PPL	LEA
k_{ass} ($M^{-1}s^{-1}$)	8.5×10^{3}	6.6×10^{3}
k_{diss} (s^{-1})	2.8×10^{-3}	5.8×10^{-3}
Ka (M^{-1})	3.0×10^{6}	1.1×10^{6}

Fig. 4 ポリラクトサミン鎖とグリコホリンの糖鎖構造



Gal GlcNAc

A 直鎖型ポリラクトサミン構造



B ウシグリコホリン主要糖鎖の推定構造

Ⅲ．研究成果の刊行に関する一覧表

Ⅳ．研究成果の刊行物・別刷り

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
H. Akiyama, T. Toida, S. Sakai, Y. Amakura, K. Kondo, Y. Konishi, T. Maitani	Determination of cyanide and thiocyanate in Sugihiratake mushroom using HPLC method with fluorometric detection	J. Health Science	52	73-77	2006
Y. Amakura, K. Kondo, H. Akiyama, H. Ito, T. Hatano. T. Yoshida, T. Maitani,	Long-chain fatty acid composition of Pleurocybella porrigens	J. Health Science *submitted*			
Y. Amakura, K. Kondo, H. Akiyama, H. Ito, T. Hatano. T. Yoshida, T. Maitani	Conjugated ketonic fatty acids from Pleurocybella porrigens	Chem. Pharm. Bull. *submitted*			

Determination of Cyanide and Thiocyanate in Sugihiratake Mushroom Using HPLC Method with Fluorometric Detection

Hiroshi Akiyama,*,[a] Toshihiko Toida,[b] Shinobu Sakai,[b] Yoshiaki Amakura,[a] Kazunari Kondo,[a] Yoshiko Sugita-Konishi,[a] and Tamio Maitani[a]

[a]National Institute of Health Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan and [b]Graduate School of Pharmaceutical Sciences, Chiba University, 1–33, Yayoi-cho, Inage-ku, Chiba 263–8522, Japan

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A novel type of encephalopathy occurred in patients with chronic kidney diseases, which was associated with the ingestion of the Sugihiratake mushroom during the fall of 2004 in Japan. We attempted to investigate whether cyanide and thiocyanate are present in the Sugihiratake samples and determined the cyanide and thiocyanate levels in fifteen samples collected from different Japanese districts using HPLC with fluorometric detection. The cyanide ions and thiocyanate ions were detected in the ranges of N.D.–114.0 and N.D.–17.0 μg/g in the samples, respectively. This is the first study to quantitatively detect cyanide and thiocyanate in the Sugihiratake mushrooms. This result demonstrated that cyanide exposure could occur from the intake of Sugihiratake mushrooms in one's diet. Furthermore, we discussed the possible association between cyanide and the onset of encephalopathy.

Key words —— Sugihiratake, cyanide, thiocyanate, HPLC, encephalopathy

INTRODUCTION

Sugihiratake is the fungus *Pleurocybella porrigens*, which is a flat mushroom that grows on cedar and pine trees during the fall season, not only in the districts of northern Japan, but is also widely distributed in Japan.[1] It has a specific flavor, and many Japanese have been favorably consuming it in the processed foods of the highly popular miso (fermented bean paste soup) and the deep-fried food tempura. However, during the fall of 2004 in Japan, an outbreak of serious encephalopathy exclusively occurred in patients with chronic kidney diseases after the intake of this mushroom in many areas of Japan including the Akita, Yamagata, and Niigata Prefectures. Therefore, there have been some reports based on the clinical findings that encephalopathy was induced after the ingestion of this mushroom. The exact factors that induced the encephalopathy remain unclear and the association between the Sugihiratake mushroom intake and the onset of this novel type of encephalopathy is still currently controversial.

In the present study, we attempted to investigate the cyanide contents in wild Sugihiratake collected from several districts in Japan using a specific HPLC method with fluorometric detection, and were the first to detect cyanide in some of these samples. In addition, we discussed the possible association between cyanide intake and the onset of encephalopathy.

MATERIALS AND METHODS

Materials —— The Sugihiratake mushroom samples were collected from the local health environment centers and the prefectural institutes of the public health and environmental science in Japan through the Ministry of Health, Labor and Welfare (MHLW) of Japan.

Reagents —— A standard solution of potassium cyanide (0.1 M) was prepared by dissolving potassium cyanide (Wako Pure Chemicals, Osaka, Japan) in 0.1 M sodium hydroxide; the concentration of cyanide was calibrated by titration with silver nitrate using potassium iodide as the indicator according to the Liebig-Dènigès method.[2,3] A standard solution of potassium thiocyanate (Wako Pure Chemicals) was prepared using redistilled water. All other

*To whom correspondence should be addressed: National Institute of Health Sciences, 1–18–1, Kamiyoga, Setagaya-ku, Tokyo 158–8501 Japan. Tel.: +81-3-3700-9397; Fax: +81-3-3707-6950; E-mail: akiyama@nihs.go.jp

Table 1. Cyanide and Thiocyanate Contents of the Sugihiratake Mushroom Samples

Sample No.	Producing district	CN^- (μg/g dry weight)	SCN^- (μg/g dry weight)
1	Akita 1	12.7	1.1
2	Akita 2	25.5	4.6
3	Yamagata	0.7	0.2
4	Niigata 1	1.8	0.5
5	Niigata 2	56.2	17.0
6	Mie	3.1	1.6
7	Gifu 1	22.1	10.3
8	Gifu 2	114.0	9.4
9	Fukui 1	3.0	1.1
10	Fukui 2	0.9	1.4
11	Ishikawa	N.D.	0.1
12	Kyoto	1.2	0.2
13	Ibaraki	96.6	8.4
14	Fukushima 1	0.6	0.1
15	Fukushima 2	0.3	N.D.

N.D.: not detected.

chemicals were of analytical reagent grade.

Preparation of Sample Solution —— The freeze-dried Sugihiratake sample was ground to a fine powder using a grinder (Retsch GmbH, Haan, Germany), and a 500 mg test sample was extracted with 10.0 ml of 0.1 M sodium hydroxide by shaking overnight in a 50 ml centrifuge tube. A one ml portion was then placed in the outer well of the Conway cell and 1.0 ml of 0.1 M sodium hydroxide was placed in the center chamber. The Conway cell and ground-glass cover were coated with silicone grease, and a glass cover was placed on top of the microdiffusion cell, leaving a small space for the addition of the acidic solution. To the samples described above for the determination of cyanide, 1.0 ml of 1.2 M sulfuric acid was added to the outer chamber. Subsequently, the ground-glass cover was moved to seal the microdiffusion cell. These cells were carefully rotated in order to mix the solution in the outer chamber. The cells were then rotated every 30 min. Cyanide in the sample was allowed to diffuse for 4 hr at room temperature and the liberated hydrogen cyanide was absorbed into the sodium hydroxide solution in the center chamber. An aliquot from the center chamber solution was analyzed by HPLC.[4] For the recovery of thiocyanate from each sample during the pretreatment procedure, the Conway microdiffusion cell was kept for 24 hr and the collected thiocyanate in the center chamber was analyzed by HPLC. Using the pretreatment procedure described above, the spiked standard cyanide and thiocyanate at the 1 μmol level were recovered at 100.2 ± 3.2 and 95.4 ± 5.5%, respectively.

HPLC conditions[4] —— The HPLC system consisted of a double-plunger pump (PU-1580, Jasco., Tokyo, Japan), an intelligent fluorescence detector (FP-920S, Jasco) with a xenon lamp and 12-μl flow cell, a chromato-integrator (D-2500, Hitachi, Tokyo, Japan) and a sample injector (7725i, Reodyne, CA, U.S.A.). The HPLC conditions were as follows: column, a strong base anion exchange resin, TSK-Gel SAX (150 × 6 mm i.d., Tosoh Co., Tokyo, Japan); eluent, 0.1 M sodium acetate buffer (pH 5.0) containing 0.2 M sodium perchlorate (flow-rate, 1.0 ml/min); chlorination reagent, 0.1% chloramines T aqueous solution (flow-rate, 0.5 ml/min); pyridine-barbituric acid reagent, a mixture of barbituric acid (1.5 g), pyridine (15 ml), concentrated hydrochloric acid (3 ml) and redistilled water (82 ml) (flow-rate, 0.5 ml/min); the excitation and emission wavelengths of the detector were 583 and 607 nm, respectively.

RESULTS AND DISCUSSION

We attempted to investigate the cyanide content in the Sugihiratake samples and determined the cyanide in fifteen samples collected from different Japanese districts using HPLC with fluorometric detection. As shown in Table 1, we detected the cyanide ions and thiocyanate ions in the ranges of N.D.–

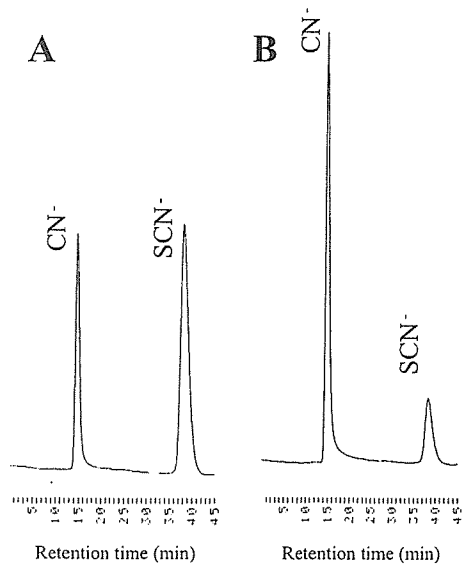

Fig. 1. Typical HPLC Chromatograms of Cyanide Ion and Thiocyanate Ion in a Sugihiratake Mushroom Sample
A; CN^- and SCN^- Standard (10 mM, respectively), B; Sample 5 (Niigata).

114.0 μg/g dry weight and N.D.–17.0 μg/g dry weight, respectively. These levels would not be lethal doses for acute toxicity even if 1 kg of the maximum level sample was consumed, because the lethal dose of cyanide is estimated to be 200–300 mg for an adult human. This result demonstrates that cyanide and thiocyanite exposures would occur from the intake of Sugihiratake mushrooms.

As for the determination of cyanide, the conventional spectrophotometric method has been previously used in forensic toxicology and waste water regulation.[5] However, it is known that thiocyanate could cause a serious false positive using the conventional method. Therefore, even forensic scientists have often mistakenly estimated cyanide because of the false positive caused by thiocyanate.[6] In the present study, we used the specifc and sensitive HPLC method of Toida *et al.*[4] after the pretreatment using the Conway cell. As shown in Fig. 1, we could simultaneously detect cyanide and thiocyanate using the HPLC system with an ion chromatographic column with non-interference determination. To our knowledge, this is the first report to accurately determine the content of cyanide and thiocyanate in Sugihiratake mushrooms. Since we confirmed the non-production of cyanide from linamarin, glucosidic cyanogens, using alkali solution (data not shown), we consider that the cyanide detected in the Sugihiratake samples would be present in the sodium form or potassium form.

Many food plants including agriculturally important species, such as cassava, flax, sorghum, alfalfa, peaches, almonds, and beans, are known to be cyanogenic.[7] Center African cassava flour contains sufficient quantities of cyanogens. When cassava is the staple part of the diet, the human daily consumption is equivalent to about one-half the lethal dose, which probably is thought to be the reason for the widespread and chronic neurological disorders called "konzo" found in this area.[7] In addition, the cyanide production has been observed in a wide range of fungi, such as *Phaeolepiota aurea*, *Rozites caperatus*, *Leucopaxillus giganteus*, and *Pleurocybella porringens* (Sugihiratake),[8] although there are no reports to describe the cyanide content in these fungi. Some reports suggested that the cyanide production in fungi could be associated with snow mold disease and fairy ring disease in some plants.[9]

To date, some clinical case studies involving the outbreak of acute encephalopathy that occurred in Japan during the fall of 2004 have already been reported.[10–13] All the cases were involved with the intake of Sugihiratake and the patients had varying degrees of renal dysfunction. The common clinical syndrome was characterized by weakness and involuntary movements of the extremities or dysarthria at the onset of the disease and subsequent intractable focal motor seizures, resulting in the generalized status of epilepticus or a comatose state. Some brain MRI examinations revealed that diffuse lesions in the basal ganglia and multiple ringed lesions in the cerebral cortex.

While there are some studies that cyanide could induce encephalopathy.[14–19] Smith *et al.* showed that comparatively small doses of cyanide given over long intervals can produce histological changes in the central nervous system of the rat.[14] As for the clinical study, Rachinger *et al.* showed that the toxicity of cyanide caused cerebral damage, primarily to the basal ganglia in the case report of patients that attempted suicide with cyanide.[17] This symptom appears to be consistent with those cases that occurred in the Akita Prefecture of Japan.

Furthermore, a recent study showed that cyanide and thiocyanate do accumulate in haemodialysis patients due to tobacco smoking.[20] Cyanide is known to be metabolized to thiocyanate by the enzyme rhodanese. This reaction is essential to life through its detoxification of cyanide, and thiocyanate synthesis can be accelerated under cyanide-loaded conditions such as tobacco smoking.[21] In addition, a

recent study showed that the risk of cerebral infarction stroke was significantly increased in individuals having high serum thiocyanate concentrations.[22] There also is some evidence that thiocyanate enhances the action of glutamate in a subclass of neuronal glutamate receptors which are involved in the neurodegenerative disorders.[23]

These results suggested that the ingestion of cyanide from foods could also induce the accumulation of cyanide and thiocyanate in the blood of patients with chronic kidney diseases and might be associated with the onset of encephalopathy. However, since the human capacity for detoxification and the toxicity of cyanide and thiocyanate from food exposure in haemodialysis patients have not been fully investigated, a further investigation is necessary in order to elucidate the relationship between the cyanide in Sugihiratake mushrooms and the onset of a new type of encephalopathy that occurred in Japan during the fall of 2004. Nevertheless, it should be noted that there could still be other factors and other substances that caused this novel type of encephalopathy in addition to the ingestion of cyanide from Sugihiratake mushrooms.

In conclusion, we were the first to determine the cyanide content in the Sugihiratake samples collected from certain areas of Japan during the fall of 2004. In addition, we showed that some samples could contain cyanide in the range of N.D.–114.0 μg/g, and suggested that its form in the Sugihiratake samples could be the sodium or potassium salt. This finding suggested that cyanide in Sugihiratake might be associated with the onset of a novel type of encephalopathy in patients with chronic kidney diseases, which occurred in Japan during the fall of 2004.

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