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Degradations of human immunoglobulins and hemoglobin by a 60 kDa cysteine proteinase of *Trichomonas vaginalis*

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Abstract: The present study was undertaken to investigate the role of cysteine proteinase of *Trichomonas vaginalis* in escaping from host defense mechanism. A cysteine proteinase of *T. vaginalis* was purified by affinity chromatography and gel filtration. Optimum pH for the purified proteinase activity was 6.0. The proteinase was inhibited by cysteine and serine proteinase inhibitors such as E-64, NEM, IAA, leupeptin, TPCK and TLCK, and also by Hg^{2+}, but not affected by serine-, metallo-, and aspartic proteinase inhibitors such as PMSF, EDTA and pepstatin A. However, it was activated by the cysteine proteinase activator, DTT. The molecular weight of a purified proteinase was 62 kDa on gel filtration and 60 kDa on SDS-PAGE. Interestingly, the purified proteinase was able to degrade serum IgA, secretory IgA, and serum IgG in time- and dose-dependent manners. In addition, the enzyme also degraded hemoglobin in a dose-dependent manner. These results suggest that the acidic cysteine proteinase of *T. vaginalis* may play a dual role for parasite survival in conferring escape from host humoral defense by degradation of immunoglobulins, and in supplying nutrients to parasites by degradation of hemoglobin.

Key words: *Trichomonas vaginalis*, cysteine proteinase, degradation, immunoglobulins, hemoglobin

INTRODUCTION

Proteinases of *Trichomonas vaginalis* participate in attachment to epithelial cells, pathogenic change of the host tissue, cytotoxicity and immune responses in host-parasite relationship (Alderete and Garza, 1988; Arroyo and Alderete, 1989). *Trichomonas vaginalis* secretes numerous proteinases during cultivation (Lockwood *et al.*, 1987; North *et al.*, 1990; Scott *et al.*, 1995) and *in vivo*. Proteinases were detected in vaginal washes of patients infected with *T. vaginalis* and antibodies against proteinases existed both in sera and vaginal washes (Alderete *et al.*, 1991; Bózner *et al.*, 1992). On the other hand, excretory-secretory product (ESP) of *T. vaginalis* degrades human IgG, IgM, and IgA (Provenzano and Alderete, 1995). These findings suggested that proteinases of *T. vaginalis* may play an important role in immune evasion mechanism of the parasite. A purified proteinase of *T. vaginalis* ESP, an acidic proteinase, has a molecular weight of 60 kDa (Garber and Lemchuk-Favel, 1994). Recently, We reported that the charac-

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terization of a partially purified proteinase from *T. vaginalis* (Min *et al.*, 1996). Live trophozoites, whole lysates, and *T. vaginalis* ESP degraded several kinds of immunoglobulins (Min *et al.*, 1997), suggesting that *T. vaginalis* can evade host immune responses by digesting immunoglobulins. However, there still remains what types of proteinase are responsible for such phenomenon. Therefore, we purified a proteinase from *T. vaginalis* lysates, determined the enzyme family by various proteinase inhibitors, and characterized its biological properties such as the degradation of human immunoglobulins and hemoglobin.

MATERIALS AND METHODS

Cells and lysates

A KT9 isolate of *T. vaginalis* was obtained from a vaginal swab of a Korean women. Axenized parasites were cultured in a TYM medium and subcultured daily. Parasites were harvested at the log phase of the growth, sonicated in 0.1 M phosphate buffer (pH 7.0), and the soluble lysates were obtained by ultracentrifugation at 100,000 *g* for 1 hr at 4°C.

Assay of proteinase activity

Proteinase activity was determined by the method of Min *et al.* (1996). The reaction mixture containing 20 μl of the enzyme was preincubated with 40 μl of 10 mM dithiothreitol (DTT, Sigma, USA) for 5 min at room temperature and 20 μl of 1 mM N-benzoyl-prolyl-phenylalanyl-arginine-*p*-nitroanilide (Bz-Pro-Phe-Arg-Nan, Sigma) was added. Sodium phosphate buffer (0.1 M, pH 6.0) was added up to 400 μl and the reaction mixture was incubated for 1.5 hr at 37°C. Enzyme activity was measured at 405 nm using a spectrophotometer (Perkin Elmer, USA). One unit of enzyme activity was defined as an amount of enzyme which causes an increase of an absorbance change of 0.1 O.D./hr.

Purification of a proteinase from *T. vaginalis* lysate

1) Activated thiol-Sepharose 4B affinity chromatography

Lysates of *T. vaginalis* were applied to an activated Thiol-Sepharose 4B column (1.5 × 20 cm, Pharmacia, Sweden) pre-equilibrated with 0.1 M Tris-HCl (pH 7.5) containing 0.3 M NaCl and 1 mM EDTA. The bound proteins were eluted with the same buffer containing 30 mM DTT. The fraction with proteolytic activities were pooled, concentrated and dialysed.

2) Bacitracin-Sepharose 4B affinity chromatography

The active fractions of activated Thiol-Sepharose 4B chromatography were applied to a Bacitracin-Sepharose 4B column (1.0 × 10 cm, Pharmacia) pre-equilibrated with 20 mM sodium acetate buffer (pH 4.0). The bound fractions were eluted with 0.1 M Tris-HCl (pH 7.0) containing 1.0 M NaCl and 25% (v/v) propanol. The active fractions were pooled, concentrated and dialyzed.

3) Sephacryl S-200 HR gel filtration

To determine the molecular weight of a proteinase, active fractions of Bacitracin-Sepharose 4B chromatography were applied to a HiPrep Sephacryl S-200 HR gel filtration column (1.6 × 60 cm, Pharmacia) pre-equilibrated with 0.1 M sodium acetate (pH 5.5) containing 0.15 M NaCl. Proteins were eluted using the same buffer at a flow rate of 0.5 ml/min. The proteolytic fractions (1.5 ml each) were pooled, concentrated and dialyzed. The molecular weight was determined with proteins of known molecular weight (Pharmacia-LKB, Sweden) including bovine serum albumin (66 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa).

Optimum pH of a purified proteinase

The optimum pH of the proteinase activity was determined by assaying the proteinase activity at pH 4.0, 4.5, 5.0 and 5.5 in 0.1 M sodium acetate, and at pH 6.0, 6.5, 7.0, 7.5 and 8.0 in 0.1 M sodium phosphate. Procedures for the proteolytic assay were done as described above using Bz-Pro-Phe-Arg-Nan as a substrate.

Effect of proteinase inhibitors

The purified proteinase was preincubated at 37°C for 40 min with various inhibitors. The

cysteine proteinase inhibitors used in this study were iodoacetic acid (IAA, 1 mM), N-ethylenemaleimide (NEM, 1 mM) and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64, 0.1 mg/ml). An aspartic proteinase inhibitor (0.1 mg/ml pepstatin A), a metallo proteinase inhibitor (1 mM EDTA), a serine proteinase inhibitor (1 mM phenylmethyl sulfonyl fluoride (PMSF)), serine and cysteine proteinase inhibitors (1 mM N-β-tosyl-L-phenylethyl chloromethyl ketone (TPCK), 1 mM N-α-tosyl-L-lycine chloromethyl ketone (TLCK) and 0.1 mg/ml leupeptin) were also used. The effect of DTT, an activator of cysteine proteinase, and Hg^{2+} ($HgCl_2$) were also assayed. All chemicals were purchased from Sigma except for E-64 which was purchased from Boehringer Mannheim, Germany.

Degradation of immunoglobulins

To observe the degradation of immunoglobulins, the purified proteinase (1, 2 or 5 μg) was incubated with 15 μg of serum IgG, serum IgA, or secretory IgA in the presence of 10 mM DTT at 37°C for 1, 2, 5, 12, and 24 hr. The reaction was stopped by the addition of reducing sample buffer of SDS-PAGE. The degradation products of immunoglobulins were transferred to nitrocellulose paper, and immunoblot was undertaken using 1:1,000 dilution of peroxidase conjugated anti-human Fc IgG (Cappel, USA) or IgA (Cappel) as a secondary antibody.

Degradation of hemoglobin

Fifty μg of hemoglobin (Sigma) was incubated with 1, 2, 5, and 10 μg of the purified proteinase for 12 hr at 37°C in the presence of 10 mM DTT. The reaction was stopped by the addition of reducing sample buffer of SDS-PAGE. The degradation products were analyzed by SDS-PAGE.

RESULTS

A proteinase of *T. vaginalis* was purified by activated thiol-Sepharose 4B, Bacitracin-Sepharose 4B, and Sephacryl S-200 HR gel filtration column in sequence. Molecular weight of the purified proteinase was estimated as 62 kDa by Sephacryl S-200 HR and 60 kDa by SDS-PAGE (Fig. 1).

The activity of the purified proteinase was approximately 78-fold higher than that of the crude extract, and the recovery was 0.4%. Maximum activity of this purified proteinase was observed at pH 6.0. The activity of purified proteinase was inhibited by cysteine proteinase inhibitors such as E-64, NEM, IAA and leupeptin, cysteine and serine proteinase inhibitors such as TPCK and TLCK, and Hg^{2+}. On the other hand, this enzyme was activated by DTT. Other serine-, metallo-, and aspartic proteinase inhibitors such as PMSF, EDTA, and pepstatin A did not affect the activity of the enzyme (Table 1).

The purified proteinase degraded immunoglobulins in a dose- and time-dependent manner. After 1 hr of incubation, a degradation products of serum IgG, 35 kDa fragment was first observed, and heavy chain (50 kDa) was degraded into 32 and 27 kDa products after further reaction (Fig. 2).

When the degradation products of serum IgA were probed using sheep anti-human Fc IgA,

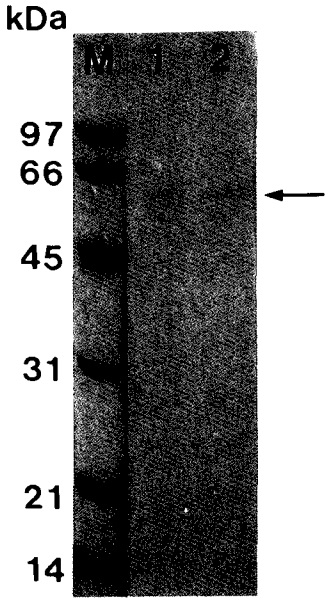


Fig. 1. SDS-PAGE (12%) analysis of a cysteine proteinase (arrow) purified from crude extract of *Trichomonas vaginalis* according to sequential chromatographic steps. M, marker; lane 1, active peaks from Bacitracin-Sepharose 4B affinity chromatography; lane 2, active peaks from Sephacryl S-200 HR gel filtration.

Table 1. Effect of inhibitors on the purified proteinase of *Trichomonas vaginalis*

		Concentration	Relative activity (%)
control			100
<Aspartic>	pepstatin A	0.1 mg/ml	99
<Metallo>	EDTA	1 mM	101
<Serine>	PMSF	0.1 mM	92
<Serine & Cysteine>	TPCK	1 mM	28
	TLCK	1 mM	25
	leupeptin	0.1 mg/ml	14
<Cysteine>	E-64	0.1 mg/ml	10
	NEM	1 mM	22
	IAA	1 mM	12
	Hg^{2+}[a]	1 mM	5
	DTT[b]	1 mM	233

[a]metal ion; [b]activator of cysteine proteinase.

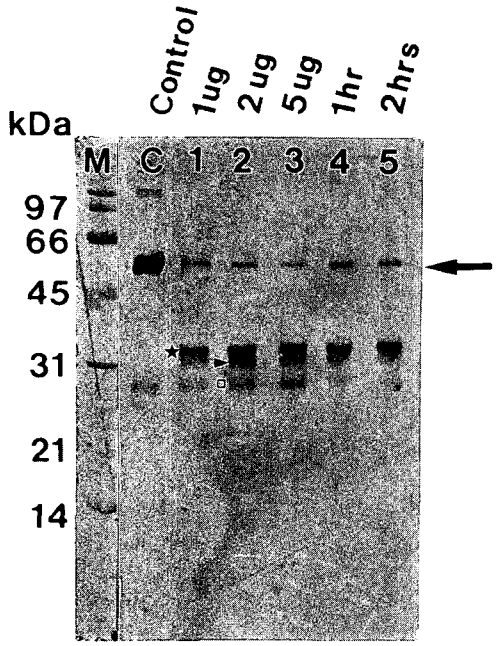


Fig. 2. Degradation of human serum IgG by the purified 60 kDa cysteine proteinase. M, markers; C, serum IgG; lane 1-3, serum IgG incubated with 1, 2 and 5 μg enzyme for 5 hr; lane 4 & 5, serum IgG incubated with 2 μg enzyme for 1 and 2 hr. SDS-PAGE (12%) of IgG and immunoblot with goat anti-human Fc IgG were done. The cysteine proteinase digested heavy chain of serum IgG in a dose-dependent manner (lane 1-3) and a time-dependent manner (lane 4 & 5). Heavy chain (→) is degraded into 35 kDa (★), 32 kDa (▶) and 27 kDa (□).

heavy chain became gradually fainter according to the incubation time in a dose-dependent manner, leaving degradation products of 48, 45, and 35 kDa. After 24 hr of incubation, it was completely digested (Fig. 3A). A similar pattern of proteolysis was observed with secretory IgA, which was degraded into 48 kDa protein. Secretory IgA was more rapidly degraded compared to serum IgG and serum IgA (Fig. 3B).

The purified enzyme also degraded dimer and monomer of hemoglobin in a dose-dependent manner, which were nearly degraded after 12 hr of incubation (Fig. 4).

DISCUSSION

Several investigators reported the degradation of immunoglobulins by live and lysates of *T. vaginalis* (Provenzano and Alderete, 1995; Min *et al.*, 1997), and the relationships between host immune response and cysteine proteinase (Plaut, 1983; Parenti, 1989). We have partially purified a 60 kDa proteinase previously (Min *et al.*, 1996) although it was mixed with other enzymes. In this study a proteinase from *T. vaginalis* lysates was purified using two affinity chromatography and gel filtration, and its biological properties such as the degradation of human immunoglobulins and hemoglobin were characterized.

The activity of this purified proteinase was maximal at pH 6.0. The enzyme activity was inhibited by E-64, IAA, NEM, leupeptin, TPCK,

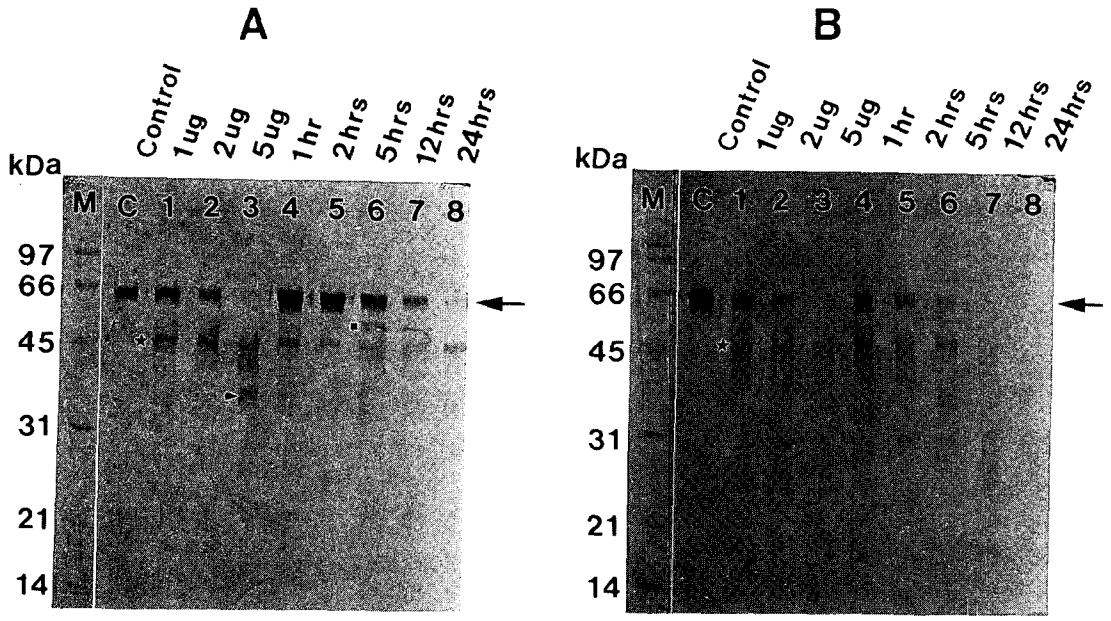
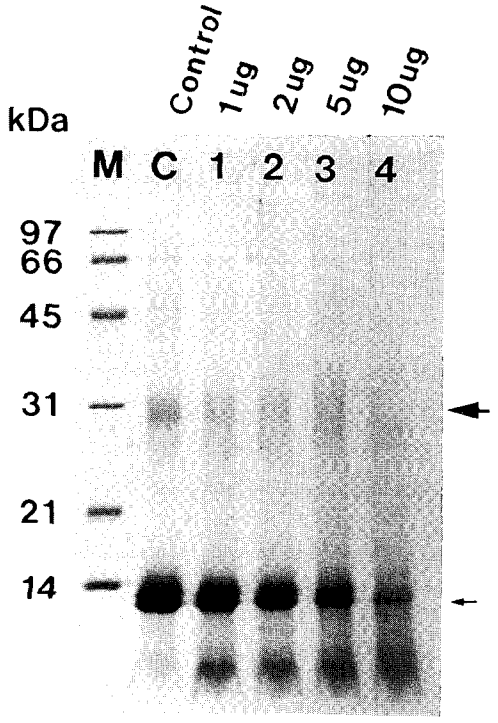


Fig. 3. Degradation of human IgA (A) and secretory IgA (B) by the 60 kDa cysteine proteinase. M, markers; C, serum IgA (A) or secretory IgA (B); lane 1-3, serum IgA (A) or secretory IgA (B) incubated with 1, 2 and 5 μg enzyme for 5 hr; lane 4-8, serum IgA (A) or secretory IgA (B) incubated with 2 μg enzyme for 1, 2, 5, 12 and 24 hr. SDS-PAGE (12%) of IgA or secretory IgA and immunoblot with sheep anti-human Fc IgA were done. The cysteine proteinase digested heavy chain of serum IgA in a dose-dependent manner (lane 1-3) and a time-dependent manner (lane 4-8). Heavy chain of serum IgA (A) (→) is degraded into 48 kDa (■), 45 kDa (★) and 35 kDa (▶), and that of secretory IgA (B) (→) is degraded into 48 kDa (★).



TLCK, and Hg^{2+}, but not by PMSF, EDTA, and pepstatin A. These results suggest that the 60 kDa enzyme of *T. vaginalis* is an acidic cysteine proteinase, which is similar to those of other parasites such as *Clonorchis sinensis*, *Schistosoma mansoni*, *Entamoeba histolytica*, and *Giardia lamblia* (Chappell and Dresden, 1986; Parenti, 1989; Song *et al.*, 1990; Avila and Calderon, 1993).

Min *et al.* (1994) demonstrated that the membrane protein with molecular weight of 60 kDa is one of the major protein of *T. vaginalis*. Previously, Garber and Lemchuk-Favel (1994) reported that a 43 kDa protease, a subunit of an extracellular protease at 60 kDa, was associated with cell surface of *T. vaginalis*. ←

Fig. 4. Degradation of hemoglobin by the 60 kDa cysteine proteinase (➡ dimer, → monomer of hemoglobin). The enzyme digested hemoglobin in a dose-dependent manner (lane 1-4). M, marker; C, control hemoglobin; lane 1-4, hemoglobin incubated with 1, 2, 5, and 10 μg enzyme for 12 hr.

Interestingly, the cysteine proteinase purified from this study and the surface antigen have the same molecular weight of 60 kDa. The cellular localization of cysteine proteinase purified from the present study should be further evaluated by immunohistochemistry or immuno-electronmicroscopic method.

IgA provides a primary immunity to parasite and bacteria localized in the gastrointestinal tract, the lung, and the urogenital system (Janeway and Travers, 1994). Secretory IgA inhibits the mucous adherence of bacteria and parasites, and an import of pathogenic material into epithelial cells (Tagliabue *et al.*, 1983). In vaginal washes of trichomoniasis patients, significantly elevated levels of IgA were found (Ackers *et al.*, 1975; Su, 1982). To escape from host immune responses, some bacteria secrete proteinases to degrade host immunoglobulins. Protozoa such as *G. lamblia*, *E. histolytica* and *T. vaginalis* also secrete cysteine proteinases which degrade the host immunoglobulins (Plaut, 1983; Parenti, 1989; Kelsall and Ravdin, 1993; Provenzano and Alderete, 1995; Min *et al.*, 1997). In this study, the purified cysteine proteinase degraded immunoglobulins, suggesting that *T. vaginalis* can escape from host immune surveillance by degrading IgA and secretory IgA. Because parasitic protozoans cause chronic disease in the host in general, protozoan cysteine proteinase may play a role in escaping from host humoral response.

Hemoglobin is used as a major nutrient by blood parasites such as *Schistosoma mansoni* and malaria, and is degraded by their proteinase into heme and globin, and the latter is subsequently degraded into free amino acids as a source of protein biosynthesis in parasites (Chappell and Dresden, 1986; Salas *et al.*, 1995). The purified cysteine proteinase of *T. vaginalis* also degraded both the monomeric and dimeric forms of hemoglobin, implying that *T. vaginalis* may use the hemoglobin as a nutrient.

Iron is an essential nutrient to the trichomonads and potentially could be acquired from hemoglobin following hemolysis (Dailey *et al.*, 1990). When hemoglobin as a sole source of iron is added to the growth medium under iron-limiting conditions, *T. vaginalis* showed excellent growth than the iron-chelated culture medium control (Alderete *et al.*, 1992). In addition, iron mediates resistance of *T. vaginalis* to complement lysis and regulates growth of trophozoites and cytoadherence, which is associated with pathogenicity of *T. vaginalis* (Lehker *et al.*, 1991; Lehker and Alderete, 1992; Alderete *et al.*, 1995). Therefore, ability of degrading hemoglobin may be related with immune evasion and pathogenicity of *T. vaginalis* other than nutrient supply to trophozoites.

From this study, we hypothesized that a 60 kDa cysteine proteinase of *T. vaginalis* play roles not only in escape from host humoral response, but also in the supply of nutrients to parasite.

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초록=

질편모충의 60 kDa 시스테인 단백분해효소의 인체 면역글로불린 및 헤모글로빈 분해능

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한양대학교 의과대학 기생충학교실 및 의과학연구소[1] 및 건국대학교 이과대학 생물학과[2]

질편모충의 시스테인 단백분해효소가 숙주-기생충 관계에서 어떤 역할을 하는지 알아보기 위해 질편모충을 대량배양하여 초음파분쇄한 후 초원심분리하여 조추출물을 얻었고, activated Thiol-Sepharose 4B, Bacitracin Sepharose affinity chromatography, Sephacryl S-200 HR gel filtration 등을 이용하여 단백분해효소를 정제하였다. SDS-PAGE로 정제도를 확인하여 이들 효소의 생화학적 특성, 그리고 인체 면역글로불린 및 헤모글로빈 분해능을 관찰하였다. 정제된 단백분해효소는 0.1 M sodium phosphate (pH 6.0)에서 최적 활성을 나타내었으며, SDS-PAGE에서 분자량은 60 kDa이었고 gel filtration에서 native 분자량은 62 kDa이었다. 정제된 단백분해효소는 시스테인 계열 억제제인 E-64, IAA, NEM에 의해서 활성이 억제되었으며, 메탈로, 세린, 아스파틱 계열 억제제에 의해서는 활성이 억제되지 않았다. Hg^{2+} 이온에 의해 활성이 억제되었고 시스테인 계열 활성제인 DTT에 의해서는 2배 이상의 활성을 보여 정제된 단백분해효소가 시스테인 계열임을 알 수 있었다. 정제된 단백분해효소와 serum IgG, serum IgA, secretory IgA를 반응시켰을 때 면역글로불린이 분해되었고 헤모글로빈과 반응시켰을 때도 헤모글로빈을 분해하였다. 이상의 결과로 보아 질편모충에서 정제한 60 kDa acidic 시스테인 단백분해효소는 인체의 IgG, IgA 및 헤모글로빈 등을 분해하여 숙주의 면역기작을 회피하며 영양대사에 이용할 것으로 생각된다.

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