Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : April 4, 2017
Accepted : May 23, 2017
Published online : June 30, 2017

Original article

The effectiveness of the peel extract of water chestnut (*Trapa bispinosa* Roxb.) in an α-crystallin glycation model with glyoxal

Tomohiro Uemura, Shouko Takeshita, Michio Yamada,

Hayashikane Sangyo Co.,Ltd., Yamaguchi, Japan

Abstract

***Objective*:** The formation of advanced glycation end products (AGEs) in lens proteins is hypothesized to be a key pathogenic mechanism for developing cataracts due to aging or diabetes. Therefore, inhibiting the glycation of lens proteins is thought to be a useful means of preventing and suppressing the progression of cataracts. Consequently, in order to verify the usefulness of peel extract of water chestnut (*Trapa bispinosa* Roxb.) (TBE) in preventing and suppressing the progression of cataracts, we created an *in vitro* model of α-crystallin —the main protein component of lenses— glycation with glyoxal and assessed its effect on the generation of N^{ε}-(carboxymethyl) lysine (CML). We also compared its activity with aminoguanidine (AG), a positive control.

***Methods*:** A mixed solution of glyoxal and bovine lens-derived α-crystallin was allowed to react for 16 h at 37°C, and afterwards the CML concentration was measured using the ELISA method. The CML concentration after TBE or AG was added was expressed as a relative value with the control value defined as 100. Additionally, an IC_{50} value was calculated for the inhibition of CML generation.

***Results*:** Glyoxal and α-crystallin were allowed to react for 16 h. When the CML concentration was compared to that before the reaction, it had markedly increased. Comparing the effect of TBE on CML generation with that of the control revealed that TBE inhibited CML generation in a concentration-dependent manner. TBE showed an IC_{50} value (μg/mL) of 30.59, and AG showed a value of 59.51.

***Conclusion*:** TBE inhibited CML generation in a model of α-crystallin glycation with glyoxal about two times more than AG. These results suggest that TBE —a food-derived material with exceptional antiglycation activity— may be useful in preventing and suppressing the progression of cataracts developed due to aging or diabetes. In the future, we will continue with an analysis of the active components and an efficacy evaluation of TBE *in vivo*.

***KEY WORDS*:** α-crystallin, cataract, glyoxal, N^{ε}-(carboxymethyl)lysine (CML), water chestnut (*Trapa bispinosa*)

Introduction

In the process of glycation, dicarbonyl compounds such as glyoxal are generated as reactive intermediates through a reaction between proteins and reducing sugars, and as a result, advanced glycation end products (AGEs) are formed [1]. In recent years, it has been revealed that the formation of AGEs *in vivo* is involved in pathological conditions such as cataracts occurring with old age and diabetes [2]. Cataracts refers to a disease in which the amount of light transmitted through the lens that reaches the retina declines, causing a decrease in vision, because the lens proteins, which were originally water-soluble, aggregate and become insoluble, causing clouding [2]. According to a 2010 report by the World Health Organization, cataracts are the cause of blindness in ~51% of cases worldwide; this number amounts to about 20 million people [3]. Clinical reports thus far suggest that the glycation of lens proteins is involved in the pathogenic mechanism for cataracts developed due to old age and diabetes [4-7]. It is thought that when lens proteins such as α-crystallin are glycated, structural changes and cross-linking occur, and the proteins aggregate, ultimately leading to a decrease in the transparency of the lens [8-10]. It has been reported that various AGEs such as N^{ε}-(carboxymethyl) lysine (CML), pentosidine, N^{ε}-(carboxyethyl) lysine (CEL), and methylglyoxal hydroimidazolone 1 (MG-H1) exist in human lenses [11, 12]. In particular, that the CML concentration of lenses taken from diabetic rats and cataracts patients is

Contact Address: Tomohiro Uemura
Hayashikane Sangyo Co., Ltd.
2-4-8 Yamato-machi, Shimonoseki, Yamaguchi, 750-8608, Japan
Phone: +81-83-267-0094 Fax: +81-083-267-0192
Email: tmuemura@hayashikane.co.jp
Co-authors; Shouko T, stakesita@hayashikane.co.jp ;
Michio Y, myamada@hayashikane.co.jp

high when compared to normal cases suggests that CML is one of the AGEs involved in cataracts [13, 14]. In addition, CML is generated by a reaction between glyoxal, which is derived from the autoxidation of glucose, and protein lysine residues [15, 16]. Accordingly, suppressing the generation of AGEs such as CML by inhibiting glycation due to glyoxal in lens proteins is thought to be one useful approach to prevent and suppress the progression of cataracts.

In this study, we focused on the peel of the water chestnut (*Trapa bispinosa* Roxb.), which can prevent glycation. Water chestnuts are annual aquatic plants of the *Trapaceae* family widely used as edible and medicinal plants not only in Asia but also throughout the world [17]. Water chestnuts are reported to have various physiological functions, including antioxidant [18] and antibacterial activities [19], immunomodulating effects [20], and antiulcer effects [21]. In our research to date, we have shown that the peel extract of water chestnut (*Trapa bispinosa* Roxb.; TBE) has antiglycation effects *in vitro* [22] and improves blood glucose levels after consumption *in vivo* [23]. However, the effect of TBE on the glycation of lens proteins —suggested to be related to cataracts— has not been shown. Accordingly, in this study, to verify the usefulness of TBE in preventing and suppressing the progression of cataracts accompanying aging and diabetes, we created a model of α-crystallin glycation with glyoxal and evaluated the effect of TBE on CML generation *in vitro*. Additionally, we used the AGE generation inhibitor aminoguanidine (AG) as a positive control.

Materials and Methods

Preparation of the peel extract of water chestnut (Trapa bispinosa Roxb.; TBE)

The water chestnut peel was dried, sterilized, and crushed, and afterwards extraction was performed using hot water (approximately six times the weight of the water chestnut peel). Dextrin was added to the extracted liquid so that the ratio of chestnut peel water extract to dextrin would be 67 : 33 using the dry weight. It was subsequently spray dried, and TBE was obtained. After the TBE was dissolved in a 100 mM phosphate buffered saline (PBS; pH 7.4), it was filtered (0.45 μm) and used as a specimen for measuring glycation inhibition.

Measurement of α-crystallin glycation inhibition

As specified below, the inhibition of α-crystallin glycation was evaluated by creating a glyoxal-α-crystallin model and carrying out measurements of CML.

The glyoxal- α-crystallin model

The α-crystallin glycation reaction with glyoxal was conducted using a revised version of the method of Kevin *et al* [15]. Either 10 μL of 100 mM PBS (pH 7.4) or sample solutions adjusted to various concentrations were added to 30 μL of a 100 mM PBS (pH 7.4), 50 μL of 10 mg/ml bovine lens-derived α-crystallin (Sigma, St. Louis, MO, USA), and 10 μL of a 10 mM glyoxal solution (Wako, Osaka, Japan). After combining the solutions (total 100 μL), the mixtures were allowed to react for 16 h at 37°C within a CO_2 incubator. Aminoguanidine (AG) (Wako) was used as a positive control. All the aforementioned reagents and measurement samples were concentration adjusted using 100 mM PBS (pH 7.4).

CML Measurement

An N^{ε}-(carboxymethyl) lysine enzyme-linked immunosorbent assay (ELISA) kit (CircuLex, Nagoya, Japan) was used in measuring CML. Sixty μL of an anti-CML monoclonal antibody solution was added to 60 μL each of a CML-HSA standard solution, a blank solution, and an α-crystallin glycation solution and were mixed thoroughly. After 100 μL of each mixed solution was added to a microplate with immobilized CML-BSA, they were placed on a shaker and left in an incubator at room temperature for 1 h. Then, the solution was removed. After the wells were washed four times with 200 μL of a wash buffer solution containing 0.2% Tween-20, 100 μL of horseradish peroxidase (HRP)-labeled anti-mouse IgG polyclonal antibody —the secondary antibody— was added to each well, and this was left in an incubator at room temperature for 1 h after being placed on a shaker. After the reaction was complete, the wells were washed as described above. One hundred μL of a solution containing tetra-methylbenzidine (TMB) was added to each well and left in an incubator at room temperature for 1 h after being placed on a shaker. After the reaction was complete, the wells were washed as described above. After 100 μL of a substrate solution was added, they were placed in an incubator at room temperature for 10 – 20 minutes after being placed on a shaker. After this, 100 μL of a reaction-stopping solution containing 1 N of sulfuric acid was added. The absorbance (wavelength: 450 nm) of each well was measured using a microplate reader (Tecan Infinite 200; Männedorf, Switzerland). The CML concentration in the α-crystallin glycation solution was calculated based on the CML-HSA standard curve. The CML concentration after the addition of each measurement sample was expressed as a relative value with the control value defined as 100. The inhibition rate of CML generation for the measurement samples was calculated as follows so that IC_{50} values could also be calculated:

Inhibition rate of CML generation (%) =
(1–CML concentration when sample added ÷ control CML concentration) ×100

Results

Changes in the CML concentration in the glyoxal-α-crystallin model are shown in ***Fig. 1***. When compared to before the reaction (0 h), the CML concentration had markedly increased after the 16-h reaction. On the other hand, for α-crystallin and glyoxal alone, very little CML was generated. Next, the effect of each material on CML generation is shown in ***Fig. 2***. CML generation was inhibited in a concentration-dependent manner with the addition of TBE or AG. The IC_{50} value (μg/mL) for CML generation inhibition was 30.59 for TBE and 59.51 for AG (***Table 1***).

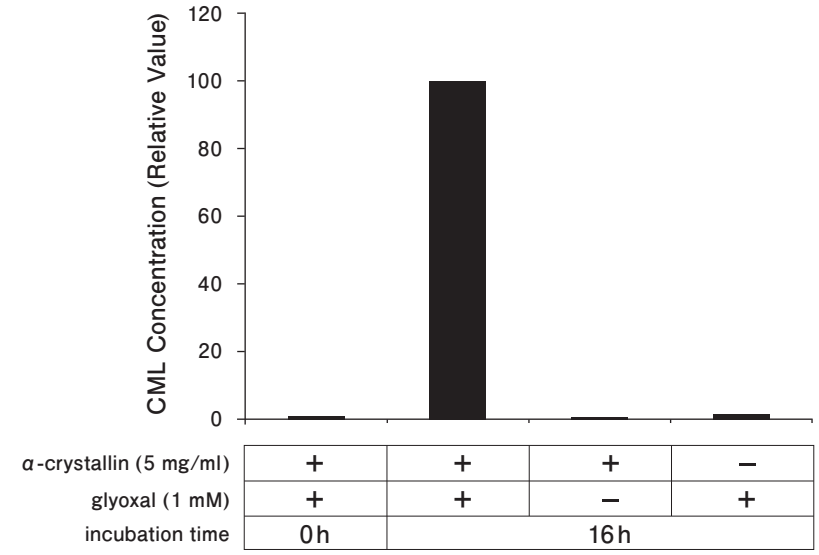

Fig. 1. Changes in CML concentration in the glyoxal-α-crystallin model before and after 16 h of incubation at 37°C. CML, N^{ε}-(carboxymethyl)lysine.

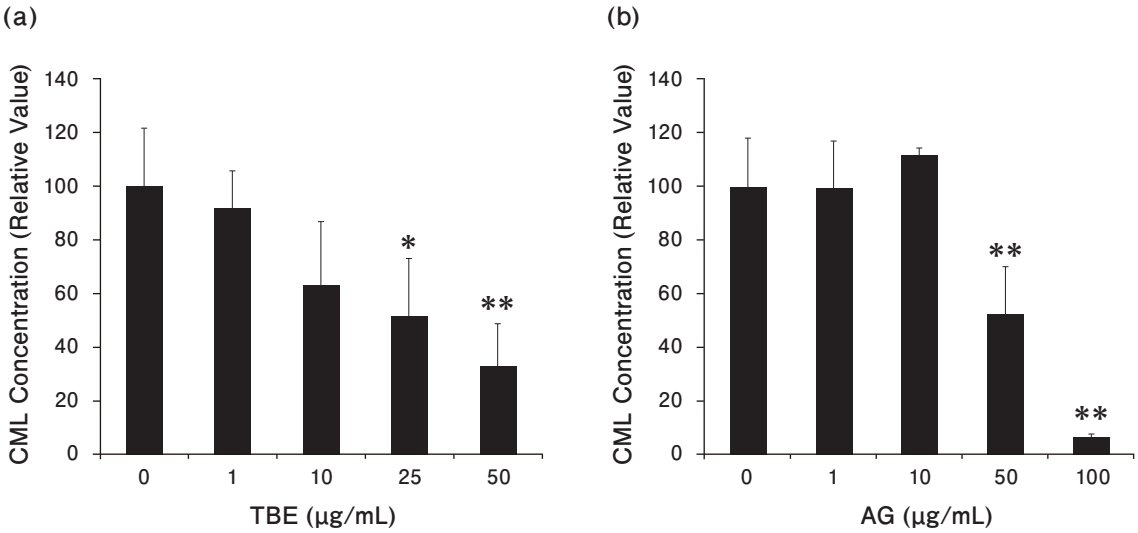

***Fig. 2.* The effects of TBE (a) and AG (b) on CML generation.**

Results are expressed as means ± SD. * $p<0.05$, ** $p<0.01$, vs. control by Dunnet's test (n = 4). TBE, water chestnut (*T. bispinosa*) peel extract; AG, aminoguanidine; CML, N^{ε}-(carboxymethyl)lysine; SD, standard deviation.

Table 1. IC_{50} values of TBE and AG for inhibition of CML generation.

Sample	IC_{50} (μg/mL)
TBE	30.59
AG	59.51

IC_{50}, 50% inhibitory concentration; TBE, water chestnut (*T. bispinosa*) peel extract; AG, aminoguanidine; CML, N^{ε}-(carboxymethyl)lysine.

Discussion

The glycation of lens proteins is thought to be one pathogenic mechanism for cataracts developed due to old age and diabetes [4-7]. In this study, in order to verify the usefulness of the peel extract of water chestnut (TBE) in preventing and suppressing the progression of cataracts, we created an α-crystallin glycation model with glyoxal and assessed the effect of TBE on CML generation *in vitro*. Glyoxal, used as an α-crystallin glycation agent, is one type of dicarbonyl compound generated in the glycation reaction process. In addition to the generation of CML, it is also involved in the cross-linking of glyoxal-lysine dimer (GOLD) [24], and it is suggested as one cause of the decreased function of biological proteins [25] and aggregation of lens proteins [26]. As such, glyoxal is one important target for AGE generation inhibitors.

In order to confirm the inhibitory activity of TBE on α-crystallin glycation, we measured CML. As a result, compared to the control, TBE inhibited CML generation in a concentration-dependent manner. Further, regarding IC_{50} values, when compared to AG [27] —an AGE generation inhibitor that traps dicarbonyl compounds such as glyoxal— TBE exhibited approximately twice the glycation inhibiting activity. To date, we have confirmed that TBE inhibits the glycation of human serum albumin *in vitro* [22], and here, we revealed the novel antiglycation effect of TBE on α-crystallin. Regarding this function of TBE, we believe it is not only due to trapping glyoxal in the same way as AG. In the past, we confirmed that TBE cleaves dicarbonyl compounds *in vitro* [22] and that this activity was stronger than that of the AGE breaker, *N*-phenacylthiazolium bromide (PTB) [28]. Additionally, components that exhibit various physiological effects, such as antioxidant effects [29], α-glucosidase inhibition [29], and immunostimulatory functions [30], have been identified in the peels of plants in the *Trapaceae* family, of which water chestnuts are a member. It is thought that the TBE also includes several of these active ingredients. From these facts, it is hypothesized that, in addition to trapping glyoxal, TBE may have degraded glyoxal, producing the striking inhibitory effect of TBE on CML generation.

AG, used as a positive control in this study, is an AGE generation inhibitor reported from the very early stages, which suppresses diabetes complications, such as nephropathy and cataracts in animal models of diabetes [27, 31]. However, AG exhibits side effects in clinical trials and has not been put to practical use [32]. Even now, AGE inhibitors such as pyridoxamine are being developed with the goal of treating diabetes complications; however, some of these drugs have been confirmed to have side effects in clinical trials [33]. On the other hand, water chestnut peel, the raw material for TBE has come to be widely used in both food and medicine [34-36]. Consequently, as a food-derived material with excellent anti-glycation properties and a low risk of side effects, it is hoped that TBE will be widely used in preventing and suppressing the progression of diabetes complications such as cataracts.

Because TBE was a stronger inhibitor of CML generation in α-crystallin than AG in this study, it is suggested that TBE may be useful in preventing and suppressing the progression of cataracts accompanying old age and diabetes. In the future, we would like to proceed with an analysis of the antiglycation components and an efficacy evaluation using animals and humans.

Conclusion

In order to verify the usefulness of TBE in the prevention and suppression of the progression of cataracts, we created an *in vitro* model of α-crystallin glycation using glyoxal and assessed the effectiveness of peel extract of water chestnut (TBE) on CML generation. When TBE was compared to the positive control AG, it markedly inhibited CML generation. It is suggested that TBE may be useful in preventing and suppressing the progression of cataracts accompanying old age and diabetes as a food-derived material with excellent antiglycation activity. In the future, we would like to proceed with an analysis of the active ingredients and an efficacy evaluation of TBE *in vivo*.

Conflicts of interest statement

This work was supported by Hayashikane Sangyo Co., Ltd.

References

1) Singh VP, Bali A, Singh N, et al. Advanced glycation end products and diabetic complications. Korean J Physiol Pharmacol. 2014; 18: 1-14.
2) Pescosolido N, Barbato A, Giannotti R, et al. Age-related changes in the kinetics of human lenses: Prevention of the cataract. Int J Ophthalmol. 2016; 9: 1506-1517.
3) World Health Organization (WHO). Prevention of Blindness and Visual Impairment. Priority eye diseases: Cataract. http://www.who.int/blindness/causes/priority/en/index1.html. Accessed March 23, 2017.
4) Garlick RL, Mazer JS, Chylack LT Jr, et al. Nonenzymatic glycation of human lens crystalline: Effect of aging and diabetes mellitus. J Clin Invest. 1984; 74: 1742-1749.
5) Araki N, Ueno N, Chakrabarti B, et al. Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. J Bio Chem. 1992; 267: 10211-10214.
6) van Boekel MA, Hoenders HJ. Glycation of crystallins in lenses from aging and diabetic individuals. FEBS Lett. 1992; 314: 1-4.
7) Balog Z, Klepac R, Sikic J, et al. Protein carbonylation and glycation in human lenses. Coll Antropol. 2001; 25: 145-148.
8) Beswick HT, Harding JJ. Conformational changes induced in lens α- and γ-crystallins by modification with glucose 6-phosphate. Implications for cataract. Biochem J. 1987; 246: 761-769.

9) Luthra M, Balasubramanian D. Nonenzymatic glycation alters protein structure and stability. A study of two eye lens crystallins. J Bio Chem. 1993; 268: 18119-18127.
10) Kumar MS, Reddy PY, Kumar PA, et al. Effect of dicarbonyl-induced browning on α-crystallin shaperone-like activity: Physiological significance and caveats of *in vitro* aggregation assays. Biochem J. 2004; 379: 273-282.
11) Tessier F, Obrenovich M, Monnier VM. Structure and mechanism of formation of human lens fluorophore LM-1. Relationship to vesperlysine A and the advanced Maillard reaction in aging, diabetes, and cataractogenesis. J Bio Chem. 1999; 274: 20796-20804.
12) Smuda M, Henning C, Raghavan CT, et al. Comprehensive analysis of Maillard protein modifications in human lenses: Effect of age and cataract. Biochemistry. 2015; 54: 2500-2507.
13) Swamy-Mruthinti S, Shaw SM, Zhao HR, et al. Evidence of a glycemic threshold for the development of cataracts in diabetic rats. Curr Eye Res. 1999; 18: 423-429.
14) Franke S, Dawczynski J, Strobel J, et al. Increased levels of advanced glycation end products in human cataractous lenses. J Cataract Refract Surg. 2003; 29: 998-1004.
15) Wells-Knecht KJ, Zyzak DV, Litchfield JE, et al. Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry. 1995; 34: 3702-3709.
16) Ferreira AE, Ponces Freire AM, Voit EO. A quantitative model of the generation of N^{ε}- (carboxymethyl) lysine in the Maillard reaction between collagen and glucose. Biochem J. 2003; 376: 109-121.
17) Adkar P, Dongare A, Ambavade S, et al. *Trapa bispinosa* Roxb.: A review on nutritional and pharmacological aspects. Adv Pharmacol Sci. 2014; 2014: 959830.
18) Kim BJ, Kim JH, Kim HP, et al. Biological screening of 100 plant extracts for cosmetic use (II): Anti-oxidative activity and free radical scavenging activity. Int J Cosmet Sci. 1997; 19: 299-307.
19) Rahman M, Wahed II, Biswas HU, et al. *In vitro* antibacterial activity of the compounds of *Trapa bispinosa* Roxb. Journal of Medical Sciences. 2001; 1: 214-216.
20) Patel S, Banji D, Banji OJF, et al. Scrutinizing the role of aqueous extract of *Trapa bispinosa* as an immunomodulator in experimental animals. Int J Res Pharm Sci. 2010; 1: 13-19.
21) Kar DM, Maharana L, Si SC, et al. Anti ulcer activity of ethanolic extract of fruit of *Trapa bispinosa* Roxb in animals. Der Pharmacia Lettre. 2010; 2: 190-197.
22) Takeshita S, Yagi M, Uemura T, et al. Peel extract of water chestnut (*Trapa bispinosa* Roxb.) inhibits glycation, degrades α-dicarbonyl compound, and breaks advanced glycation end product crosslinks. Glycative Stress Res. 2015; 2: 72-79
23) Takeshita S, Ishioka Y, Yagi M, et al. The effects of water chestnut (*Trapa bispinosa* Roxb.) on the inhibition of glycometabolism and the improvement in postprandial blood glucose levels in humans. Glycative Stress Res. 2016; 3: 124-132.
24) Frye EB, Degenhardt TP, Thorpe SR, et al. Role of the Maillard reaction in aging of tissue proteins: Advanced glycation end product-dependent increase in imidazolium cross-links in human lens proteins. J Bio Chem. 1998; 273: 18714-18719.
25) Voziyan P, Brown KL, Chetyrkin S, et al. Site-specific AGE modifications in the extracellular matrix: A role for glyoxal in protein damage in diabetes. Clin Chem Lab Med. 2014; 52: 39-45.
26) Argirova M, Breipohl W. Comparison between modifications of lens proteins resulted from glycation with methylglyoxal, glyoxal, ascorbic acid, and fructose. J Biochem Mol Toxicol. 2002; 16: 140-145.
27) Thornalley PJ. Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. Arch Biochem Biophys. 2003; 419: 31-40.
28) Vasan S, Zhang X, Zhang X, et al. An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. Nature. 1996; 382: 275-278.
29) Yasuda M, Yasutake K, Hino M, et al. Inhibitory effects of polyphenols from water chestnut (*Trapa japonica*) husk on glycolytic enzymes and postprandial blood glucose elevation in mice. Food Chem. 2014; 165: 42-49.
30) Ramsankar S, Chanchal K. Nandan, Ipsita K. Sen, et al. Structural studies of an antioxidant, immunoenhancing polysaccharide isolated from the kernel of *Trapa bispinosa* fruit. Journal of Carbohydrate Chemistry. 2012; 31: 686-701.
31) Swamy-Mruthinti S, Green K, Abraham EC. Inhibition of cataracts in moderately diabetic rats by aminoguanidine. Exp Eye Res. 1996; 62: 505-510.
32) Freedman BI, Wuerth JP, Cartwright K, et al. Design and baseline characteristics for the aminoguanidine clinical trial in overt type 2 diabetic nephropathy (ACTION 2). Control Clin Trials. 1999; 20: 493-510.
33) Nenna A, Nappi F, Avtaar Singh SS, et al. Pharmacologic approaches against advanced glycation end products (AGEs) in diabetic cardiovascular disease. Res Cardiovasc Med. 2015; 4: e26949.
34) Tanaka T. Tanaka's cyclopedia of edible plants of the world. 1st ed, pp731, Keigaku Pub Co, Tokyo, 1976.
35) Arima S. Studies on growth and yield performance of water chestnut (*Trapa bispinosa* Roxb.). Bulletin of the Faculty of Agriculture, Saga University. 1994; 76: 1-79. (in Japanese)
36) Chuuyaku-daijiten. pp2703, Shogakukan, Tokyo, 1985. (in Japanese)