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Inhibitory Activity of Corn-Derived Bisamide Compounds against α-Glucosidase

TOSHIO NIWA,*,† UMEYUKI DOI,† AND TOSHIHIKO OSAWA‡

Department of Research and Development, San-ei Sucrochemical Company, Ltd., Chita 478-8503, Japan and Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Nagoya 464-8601, Japan

Bioassay guided fractionation from corn gluten meal, a byproduct of a starch manufacturing plant, gave *N*-*p*-coumaroyl-*N*′-feruloylputrescine (**1**) and *N*,*N*′-diferuloylputrescine (**2**) as α-glucosidase inhibitors. Some structure−activity relationships were studied by comparing the inhibitory activity by preparing some related compounds, and it was revealed that the hydroxyl group was important for the inhibitory activity of bisamide alkaloids, but not the redox potential.

KEYWORDS: α-Glucosidase inhibitor; amides; corn; ferulic acid

INTRODUCTION

In a previous study, low postprandial plasma glucose concentrations were reported to be effective for both healthy (*1*) and diabetic subjects (*2*) through the consumption of legume seeds. However, the precise mechanism involved is ambiguous, although the activity was sometimes ascribed to "fiber" (*3*) or "starch particle size" (*4*). Another possibility suggested was an effect on the hydrolysis of carbohydrates (*5*), and an inhibition of α-glucosidase, which catalyzes the final step in dietary carbohydrates and seems to be useful for suppressing the postprandial glucose level. Non-insulin-dependent diabetes mellitus (NIDDM), which is caused by a secretory decrease in insulin from pancreatic Langerhans β cells or a lowering of the insulin resistance (*6*, *7*), is a serious problem in developed countries. One of the most beneficial therapies for NIDDM is said to be one that achieves optimal blood glucose control after a meal, and α-glucosidase inhibitors perform a very useful activity by delaying glucose absorption (*8*, *9*). Glucosidase inhibitors have also been very interesting in regard to some other biological activities (*10*−*13*); thus, there might be value in searching for a novel inhibitor, especially derived from a food from a safety point of view.

We have been studying the byproducts produced during the process of manufacturing starch from corn (*14*), and corn-derived materials have often been used in some different experimental studies as they have a useful activity regarding glucose tolerance (*15*). Recently, an inhibitory activity of a corn extract on α-glucosidase was reported (*16*), and we found the inhibitory activity in the extract of a byproduct, corn gluten meal (CGM). We therefore aimed to elucidate the structure of the α-glucosidase inhibitor from the CGM. The present study describes an inhibitory activity of corn-derived bisamide compounds on α-glucosidase, and some structure−activity relationships are described by synthesis of the related amide compounds.

MATERIALS AND METHODS

Instruments. HPLC analysis of phenolic compounds was accomplished by a 250 × 4.6 mm i.d. Wakosil-II 5C18 HG column (Wako Pure Chemical Industries Ltd., Osaka, Japan) on a Shimadzu CLASS-LC10 series HPLC system equipped with a SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan) at 40 °C with a flow rate of 1.0 mL/min. LC-MS was performed with the same column connected to a Fisons/VG Platform II mass spectrometer with a positive electrospray interface. The NMR spectra were measured in deuterated dimethyl sulfoxide with a Brucker AM-400 spectrometer. All chemical shifts are reported as δ values (parts per million) relative to TMS. High-resolution FAB-MS experiments were carried with a JEOL JMS-700 mass spectrometer (Tokyo, Japan). The UV spectra were recorded on a Hitachi U-2000 spectrophotometer (Tokyo, Japan). The IR spectra were measured with a Jasco 7000S infrared spectrometer (Tokyo, Japan).

Chemicals. 1-Aminobutane, benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), *p*-nitrophenyl-α-D-glucopyranoside, putrescine, and yeast α-glucosidase were purchased from Wako Pure Chemical. Cinnamic acid and *p*-coumaric acid were obtained from Nacalai Tesque Inc., Ltd. (Kyoto, Japan). Ferulic acid was a product of Acros Organics (Geel, Belgium).

Purification of α-Glucosidase Inhibitors from CGM. CGM (200 g), obtained from our plant (Aichi, Japan), was extracted with 50% aq. EtOH (600 mL) for 6 h at ambient temperature. The extract was filtered and evaporated in vacuo to remove any alcohol. The residual solution was extracted with EtOAc, then the organic phase was washed, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The EtOAc soluble fraction (0.51 g) was separated by BW-300 silica gel column chromatography

* Corresponding author. Telephone (+81)(562)55−5197. Fax (+81)-(562)55-5819. E-mail toshio-niwa@sanei-toka.co.jp.
† San-ei Sucrochemical Co., Ltd.
‡ Nagoya University Graduate School of Bioagricultural Sciences.

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(Fuji Silysia, Aichi, Japan) eluted with *n*-hexane/EtOAc/MeOH/TFA (200:200:100:1). Further purification of the corresponding fraction (157 mg) was accomplished by preparative HPLC eluted with a solvent mixture of H_2O/MeOH/TFA (550:450:1) with a flow rate of 5.0 mL/min at ambient temperature and monitored at 300 nm. *N*-*p*-Coumaroyl-*N*′-feruloylputrescine (**1**; 15.0 mg) and *N*,*N*′-diferuloylputrescine (**2**; 33.6 mg) were thus isolated as α-glucosidase inhibitors from the CGM.

Sample Preparation. Preparations of amide compounds were accomplished from the corresponding acid chloride (*17*) and BOP reagent (*18*). The procedure for *N*-*p*-coumaroyl-*N*′-feruloylputrescine synthesis is described briefly. *p*-Coumaric acid and *N*-*Boc*-1,4-diaminobutane (Fluka Chemie AG, Buchs, Switzerland) were condensed at room temperature using the BOP reagent. The protected amine was treated with TFA at ambient temperature for 30 min and the residual TFA was removed under reduced pressure. Then, the deprotected amine moiety was condensed with *O*-acetyl-ferulic acid chloride in the presence of an excess amount of triethylamine in an ice-cold bath. Finally, the acetyl group was easily hydrolyzed by aqueous 1 N NaOH. The product was in good agreement with the CGM-derived compound (12% from *p*-coumaric acid) by HPLC analysis.

*N-p-Coumaroyl-N′-feruloylputrescine (**1**)*. Colorless oil. UV λ_{max} (EtOH) nm (ϵ): 221.5 (26000), 229 (26400), 293.5 (37600), 311.5 (39400). IR ν_{max} (KBr) cm^{-1}: 3270, 1655, 1590, 1515. HR FAB-MS (positive ion) $C_{23}H_{27}N_2O_5$ ($[M+H]^+$) (Calcd. 411.1920, Found 411.2016). ^1H NMR (400 MHz) δ 9.78 (1H, s), 9.36 (1H, s), 7.94 (2H, m), 7.37 (2H, d, J = 8.6 Hz), 7.31 (2H, d, J = 15.7 Hz), 7.11 (1H, d, J = 1.8 Hz), 6.98 (1H, dd, J = 1.8, 7.8 Hz), 6.79 (1H, d, J = 7.8 Hz), 6.79 (2H, d, J = 8.7 Hz), 6.44 (1H, d, J = 15.6 Hz), 6.40 (1H, d, J = 15.6 Hz), 3.80 (3H, s), 3.18 (4H, m), 1.48 (4H, m).

Other compounds used for structure−activity relationships were also prepared from corresponding acid chloride and amine in a chloroform solution in a manner similar to that described previously. The structures were confirmed by spectroscopic methods, such as ^1H NMR, UV spectra obtained from photodiode array equipped HPLC, and mass spectra.

*N,N′-Dicinnamoylputrescine (**3**)*. White powder. HR FAB-MS (positive ion) $C_{22}H_{25}N_2O_2$ ($[M+H]^+$) (Calcd. 349.1916, Found 349.1920). ^1H NMR (400 MHz) δ 8.09 (2H, t, J = 5.6 Hz), 7.55 (4H, d, J = 6.8 Hz), 7.43−7.34 (8H, m), 6.62 (2H, d, J = 15.6 Hz), 3.20 (4H, m), 1.49 (4H, m).

*N-Feruloylaminobutane (**4**)*. Colorless oil. HR FAB-MS (positive ion) $C_{14}H_{20}NO_3$ ($[M+H]^+$) (Calcd. 250.1443, Found 250.1419). ^1H NMR (400 MHz) δ 9.37 (1H, s), 7.90 (1H, t, J = 5.6 Hz), 7.30 (1H, d, J = 15.8 Hz), 7.11 (1H, d, J = 1.8 Hz), 6.99 (1H, dd, J = 2.0, 8.4 Hz), 6.79 (1H, d, J = 8.1 Hz), 6.43 (1H, d, J = 15.8 Hz), 3.80 (3H, s), 3.16 (2H, m), 1.43 (2H, m), 1.31 (2H, m), 0.89 (3H, t, J = 7.3 Hz).

*O-Acetyl-N-feruloylaminobutane (**5**)*. White powder. HR FAB-MS (positive ion) $C_{16}H_{22}NO_4$ ($[M+H]^+$) (Calcd. 292.1548, Found 292.1642). ^1H NMR (400 MHz) δ 8.04 (1H, t, J = 5.6 Hz), 7.40 (1H, d, J = 15.8 Hz), 7.31 (1H, d, J = 1.6 Hz), 7.15 (1H, dd, J = 1.7, 8.2 Hz), 7.11 (1H, d, J = 8.1 Hz), 6.62 (1H, d, J = 15.8 Hz), 3.81 (3H, s), 3.18 (2H, m), 2.26 (3H, s), 1.45 (2H, m), 1.32 (2H, m), 0.89 (3H, t, J = 7.2 Hz).

Measurement of Inhibitory Activity on α-Glucosidase. The inhibitory activity was measured using a 96-well plate, using quercetin dihydrate (Kanto Chemical Co., Inc., Tokyo, Japan) as a positive control, as described by Watanabe et al. (*16*). In brief, yeast α-glucosidase (10 μg/mL) dissolved with 0.1 M phosphate buffer (pH 7.0) containing 0.2% bovine serum albumin and 0.02% sodium azide was used as an enzyme solution. Ten microliters of methanolic samples (2.0 mM) were added to the enzymatic solution (50 μL). After 5 min incubation, 5.0 mM of *p*-nitrophenyl-α-D-glucopyranoside (50 μL) was added. After another period of incubation for 5 min at ambient temperature, the increase in absorbance at 405 nm was measured with a model 550 microplate reader (Bio-Rad, Hercules, CA).

Antioxidative Activity Measurement. The antioxidative activity was measured by the thiobarbituric acid reactive substances (TBARS) on rabbit erythrocyte membrane ghost treated with *tert*-butyl hydroperoxide (*19*) with a slight modification. In brief, rabbit erythrocyte membrane (0.5 mL) prepared from commercially available rabbit blood, by washing with isotonic buffer solution and lysed in 10 mM phosphate buffer (pH 7.4), was treated with 25 mM *tert*-butyl hydroperoxide (50 μL) with or without samples dissolved in MeOH (2.0 mM, 100 μL). These reaction mixtures were incubated for 20 min at 37 °C, and TBARS of each sample were determined using UV absorption at 532 nm on a spectrophotometer.

Effect of Ultrafiltration on α-Glucosidase Inhibition by *N,N′*-Diferuloylputrescine (2). An enzyme solution (10 mL) was diluted by MeOH (2 mL) to avoid any insolubility problem on the inhibitor. The diluted enzymatic solution (4.5 mL) was treated with 0.5 mL of MeOH or methanolic sample solution of **2** (5.0 mM). Two milliliters of each solution was applied to a USY-1 ultrafilter (10 000 nominal molecular weight limit) (Advantec, Dublin, CA). An unfiltered high-molecular weight fraction was redissolved with 2.0 mL of the solvent mixture of MeOH/0.1 M phosphate buffer (1:3), and the recovered enzymatic activity of 50 μL was measured by adding 5.0 mM of *p*-nitrophenyl-α-D-glucopyranoside (50 μL). The enzyme solutions without ultrafiltration were also provided for measurement.

RESULTS AND DISCUSSION

Extraction of the CGM was carried out using aqueous EtOH with several concentrations. Among these solvents used, 50% aq. EtOH extract showed good results in activity at the same weight concentration (data not shown). An extract of CGM by 50% aq. EtOH was applied to the isolation procedures, and gave two active compounds. ^1H NMR of **2** had a similar spectrum to that of ferulic acid in the olefinic region; however, the molecular weight obtained from LC-MS was higher than ferulic acid, with *m*/*z* 441. From the LC-MS profile with a positive electrospray interface and the stability against alkaline hydrolysis (data not shown), we speculated that this compound had an amide moiety. With two methylene signals in the ^1H NMR spectrum, we speculated that **2** had the structure illustrated in **Figure 1**, and the spectrum was in good agreement with that previously reported (*20*). On the other hand, the molecular weight of **1** was 30 mass units smaller than that of **2** on LC-MS analysis. The ^1H NMR spectrum of **1** suggested the presence of *p*-coumaric acid and ferulic acid moieties in the structure, even though somewhat complicated in the olefinic region. We then considered that the differences derived from the loss of a methoxyl moiety of **2**. To confirm this structure, we synthesized **1** and as expected, the product had the same profiles on HPLC with photodiode array detection and LC-MS analysis.

From our previous study on the byproduct produced in our starch manufacturing plant (*14*), antioxidative activity was expected for these phenolic alkaloids. We then compared the inhibitory activity of **1**, **2**, and ferulic acid on yeast α-glucosidase, together with the membrane oxidation. As a result, *N*-*p*-coumaroyl-*N*′-feruloylputrescine (**1**) had much stronger inhibitory activity than *N,N*′-diferuloylputrescine (**2**) on α-glucosidase,

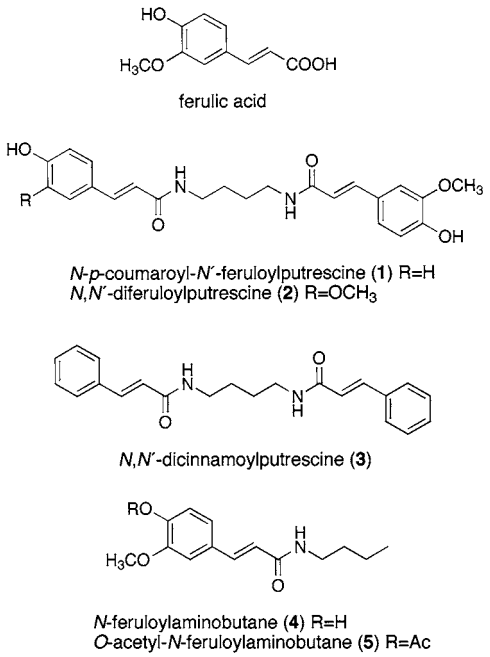

Figure 1. Structures of corn-derived α-glucosidase inhibitors and related synthetic compounds employed in this study.

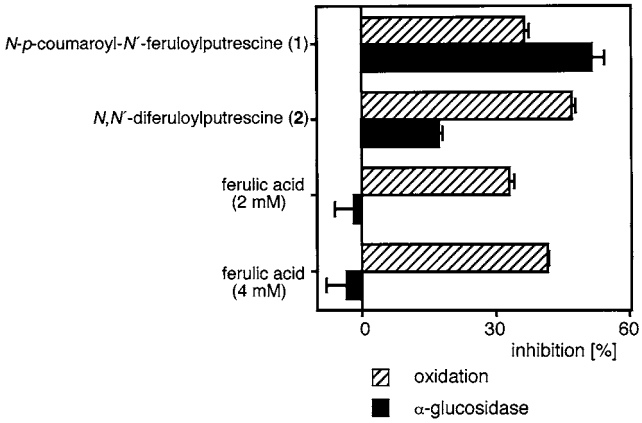

Figure 2. Inhibitory activities of corn-derived phenolic compounds on α-glucosidase and membrane oxidation. Inhibitory activity was obtained with a sample concentration at 2.0 mM by comparing the results of MeOH alone. Data represent the mean ± SD of three independent measurements.

whereas the reverse result was observed regarding their antioxidative activity (**Figure 2**). Also, ferulic acid itself, a potent antioxidant, did not show any inhibitory activity against enzymatic hydrolysis. In a previous study, Nishioka et al. (*21*) isolated *N*-*p*-coumaroyltyramine as an α-glucosidase inhibitor from *Allium* sp. They also examined some structure−activity relationships, and revealed that neither *p*-coumaric acid nor tyramine had any inhibitory activity. Moreover, they reported that *p*-coumaroyltyramine is a more potent α-glucosidase inhibitor than *N*-caffeoyltyramine, whereas caffeic acid is a more potent antioxidant than *p*-coumaric acid (*18*, *22*, *23*). From these previous results, we prepared *N*,*N*′-dicinnamoylputrescine (**3**), which had no hydrogen donatable phenolic moiety, to examine the contribution of bisamide moiety itself on the α-glucosidase. Also, *N*-feruloylaminobutane (**4**) and its acetate (**5**) were prepared (**Figure 1**), and subjected to the assay for elucidation of the relationship of bisamide moiety. By measuring the α-glucosidase inhibitory activity of the synthetic compounds together with the structurally related compounds, neither *N*,*N*′-dicinnamoylputrescine (**3**) nor putrescine itself had any inhibitory activity (**Figure 3**), although **3** had a solubility problem. These results suggested that the hydroxyl moiety of the amides was necessary for the inhibition of α-glucosidase. This result was also supported by comparison with the inhibitory effects of *N*-feruloylaminobutane (**4**) and its acetate (**5**), in which the activity of **4** was lost on *O*-acetylation. Surprisingly, the inhibitory activity of the amide compound on α-glucosidase was attenuated by the additional ferulic acid amide moiety when comparing the activity of *N*,*N*′-diferuloylputrescine (**2**) and *N*-feruloylaminobutane (**4**).

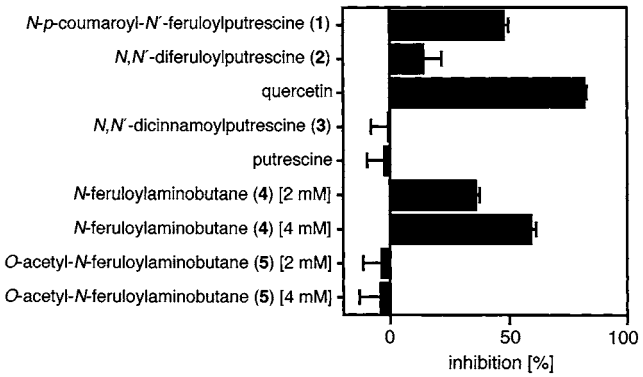

Figure 3. Inhibitory activity of amide compounds on α-glucosidase. Inhibitory activity was obtained with a sample concentration at 2.0 mM by comparing the results of MeOH alone. Data represent the mean ± SD of three independent measurements.

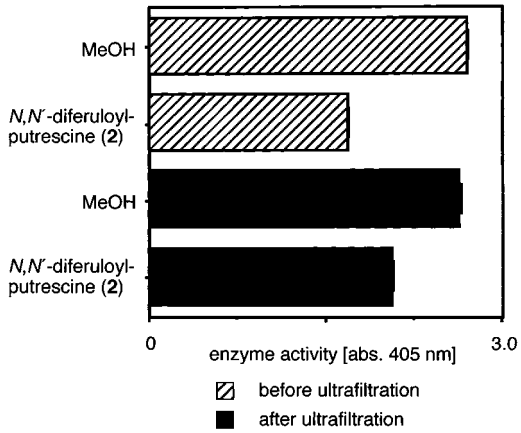

Figure 4. Recovery of enzymatic activity by elimination of *N*,*N*′-diferuloylputrescine (**2**) by ultrafiltration. The enzyme solutions, presence or absence with *N*,*N*′-diferuloylputrescine (final 0.5 mM), were treated by ultrafiltration. The unfiltered enzymes were redissolved and reacted with *p*-nitrophenyl-α-D-glucopyranoside. Data represent the mean ± SD of three-color development.

The inhibitory mechanisms of glucosidases have been explained by two distinct mechanisms: reversible inhibitors that have a high-affinity for the enzyme (*24*−*26*), and irreversible inhibitors that react with a carboxylic acid of the active site of the enzyme (*27*−*29*). The α-glucosidase inhibitors described here are not electrophilic, as are the irreversible inhibitors. We speculated that the inhibitors might interact with the enzyme, and then act as reversible inhibitors. To confirm this, we removed the unreacted inhibitor from the enzyme solution by ultrafiltration, and examined whether the activity would be recovered or not. We found that the yeast α-glucosidase did not pass through the membrane, because the unfiltered fraction retained the activity by redissolving with a same amount of buffer (**Figure 4**, MeOH), and that the low-molecular weight inhibitor (**2**) was almost removed, as shown by the HPLC

analysis (data not shown). When an enzyme solution containing **2** was treated with the ultrafiltration and then redissolved with same concentration, the α-glucosidase activity was considerably recovered (**Figure 4**). From these results, we concluded that these amide compounds are reversible inhibitors, and that substituents on the aromatic ring or some additional moieties, which affected the steric hindrance, might decrease the inhibitory activity.

Recently, *N*-*p*-coumaroyl-*N*′-feruloylputrescine (**1**) was detected in lipid extracts of corn kernel (*30*), even though the structure has not fully determined, and existence of the several hydroxycinnamic acid amides in a corn were reported (*31*, *32*). Also, some amide compounds were induced in several plants by different kinds of external stimulation (*33*−*35*), and the α-glucosidase inhibitors have been suggested to be antiviral agents (*36*). We think that the bisamides (**1** and **2**) may play a protective role in the corn kernel via the glucosidase inhibition.

ABBREVIATIONS USED

NIDDM, noninsulin-dependent diabetes mellitus; CGM, corn gluten meal; BOP, benzotriazole-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate; TBARS, thiobarbituric acid reactive substances.

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