Isolation and Identification of Peptidic α-Glucosidase Inhibitors Derived from Sardine Muscle Hydrolyzate

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We report here the isolation of α-glucosidase (AGH) inhibitory peptides derived from sardine muscle hydrolyzate, which was prepared by digestion with *Bacillus licheniformis* alkaline protease. As a result of reversed-phase HPLC purification, two AGH inhibitory peptides were isolated from a DEAE-Sephadex A-25 column eluate. The peptides were identified as follows: Val–Trp ($IC_{50} = 22.6$ mM) and Try–Tyr–Pro–Leu ($IC_{50} = 3.7$ mM). AGH inhibitory studies of Try–Tyr–Pro–Leu and its derivatives demonstrated the importance of the tri-peptide chain length as well as the hydrophobic aromatic amino acid tyrosine at the N-terminus, aliphatic amino acids at the C-terminus, as well as an amide proton from the peptide chain at the middle position of the tri-peptide to develop AGH inhibition activity.

Introduction

Developments of therapeutic drugs are under way for the prophylaxis and various clinical therapeutic treatments of the noninsulin-dependent diabetes mellitus (NIDDM) or its combined diseases such as retinopathy, neuropathy, and cataracts (Kageyama, 1996). Among them, one of the most direct and beneficial therapy for NIDDM that achieves optimum blood glucose control after a meal is the delay of glucose absorption (Bischoff, 1994; Toeller, 1994), and potent α-glucosidase (AGH, EC 3.2.1.20) inhibitors such as acarbose or voglibose fulfill these demands (Odaka *et al.*, 1992). Contrary to these synthetic inhibitors, the inhibitors from food components would be beneficial by taking into account the improvement of quality of life.

In our previous paper (Matsui *et al.*, 1996), we have examined AGH inhibition potency of various foodstuffs, in which *Bacillus licheniformis* alkaline protease hydrolyzate from sardine muscle ($IC_{50} = 48.7$ mg/ml) as well as green and oolong teas ($IC_{50} = 11.1$ and 11.3 mg/ml, respectively) strongly retarded the cleavage of *p*-nitrophenyl α-D-glucopyranoside (PNP–G) as a synthetic substrate. In addition, the hydrolyzates prepared by other proteases (pepsin, trypsin, and denazyme AP) had a distinct inhibitory activity towards AGH. This finding strongly suggests that the inhibitors in sardine muscle hydrolyzate may be peptidic compounds newly produced by action of protease, and not naturally occurring compounds.

There have been a few reports with respect to AGH inhibitors from foodstuffs, and natural polyphenolic inhibitors have been identified from Tochu-cha (Watanabe *et al.*, 1997) and oolong tea (Nakahara *et al.*, 1993). D-Xylose as a food interigent was also found to have an *in vivo* suppression effect against the increase of portal plasma glucose in Sprague-Dawley rats (Matsuura *et al.*, 1997). Contrary to these studies regarding naturally occurring inhibitors, there is no report on peptides with AGH inhibitory activity. Thus, in this study, we have tried to isolate and identify the peptidic AGH inhibitors in sardine muscle hydrolyzate by high-performance liquid chromatography (HPLC).

Materials and Methods

Materials

α-Glucosidase from bakers yeast (AGH, 9 U/mg) were purchased from Sigma Chemicals Co. (MO, U.S.A.) and synthetic substrate, *p*-nitrophenyl α-D-glucopyranoside (PNP–G) was from E. Merck

(Darmstadt, Germany). One unit is defined as the amount of AGH to hydrolyze 1 μmol of PNP–G per min at pH 6.8 at 37 °C. *Bacillus licheniformis* alkaline protease was obtained from Novo. Co. (2.4L, type FG, Chiba, Japan). All other chemicals were obtained from Nacalai Tesque (Kyoto, Japan) and used without purification.

Preparation of alkaline protease sardine muscle hydrolyzate

Sardine muscle (10 g) supplied by Senmi Ekisu Co. (Ozu, Japan) was hydrolyzed under incubation condition with 0.3 wt% protease for 17 h at 50 °C by the same procedure as described in our previous paper (Matsui *et al.*, 1996). After centrifuging at 1000 g, the supernatant was lyophylized.

Fractionation of the hydrolyzate by column chromatography

The lyophilizate (0.4 g) redissolved in 50 ml of 0.1 M phosphate buffer (pH 7.8) was applied to DEAE-Sephadex A-25 (OH⁻ form, Pharmacia LKB, Co., Tokyo, Japan) column (∅ 3.0×7.5 cm) in order to obtain more potent AGH inhibitory fractions. The active fractions eluted by a stepwise gradient (300 ml) with 0.1 and 0.3 M NaCl in 0.1 M phosphate buffer (pH 7.8) denoted as fractions B and D, respectively, were collected, and dialyzed against distilled water with a Spectra/Por membrane (<100 MW, Funakoshi Co., Tokyo, Japan).

Purification of AGH inhibitors

AGH inhibitors in the fractions B and D were then subjected to the HPLC purification. At the first step of HPLC (Shimadzu LC-10AD instrument, Kyoto, Japan), each sample was applied on a TSK gel ODS-120T column (∅ 0.78×30 cm, Tosoh Co., Tokyo, Japan) and eluted with a linear gradient of acetonitrile (5 to 50% for 45 min) at a flow rate of 1.0 ml/min. The fraction with potent AGH inhibitory activity was concentrated by the evaporation under reduced pressure. The concentrate was then put directly to a phenyl silica column ∅ 0.46×25 cm, Cosmosil 5Ph, Nacalai Tesque Co., Kyoto, Japan), and eluted with a linear gradient of acetonitrile (5 to 50% for 45 min) at a flow rate of 0.5 ml/min. Final chromatography of active fraction from a 5Ph column was performed on a C$_{18}$ column (∅ 0.46×25 cm, Cosmosil 5C$_{18}$-AR II, Nacalai Tesque Co., Kyoto, Japan), and eluted with a linear gradient of acetonitrile (18 to 21% for 30 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min.

Identification and synthesis of isolated AGH inhibitors

The amino acid composition and sequence were analyzed with a Shimadzu LC-6A amino acid analyzer, and a Shimadzu PPSQ-21 protein sequencer, respectively.

Identified peptide and its analogs were synthesized using Fmoc solid phase synthesis on a Kokusan Peptide Synthesizer (Kokusan Chemical Works Ltd., Tokyo, Japan).

Assay for AGH inhibitory activity

AGH inhibitory activity was determined by the procedure reported elsewhere (Matsui *et al.*, 1996) using PNP–G as a substrate. Briefly, 10 μl of the AGH inhibitor and 40 μl of 32 mU AGH solution in a 50 mM phosphate buffer (pH 7.0) containing 100 mM NaCl were incubated at 37 °C for 15 min after adding 950 μl of 0.7 mM PNP–G in the buffer. After being stopped by adding 1.0 ml of a 0.5 M Tris (tris(hydroxymethyl)aminomethane) solution, the absorbance of PNP released from PNP–G at 400 nm was measured. The inhibitory ratio (%) to evaluate the AGH inhibitory activity can be calculated as follows:

$$\text{Inhibitory ratio (\%)} = (A_C - A_S)/(A_C - A_B) \times 100$$

where A_C, A_S, and A_B represent the absorbance of the control, sample, and blank, respectively. The concentration of AGH inhibitor required to inhibit 50% of the AGH activity under the assay conditions is defined as the IC$_{50}$ value.

In the kinetics measurement, the final concentration of PNP–G was set in the range of 0.1 mM to 1.0 mM. The inhibition mode was determined from the Lineweaver-Burk plot.

Results and Discussion

Isolation and identification of AGH inhibitors from sardine muscle hydrolyzate

In a previous paper (Matsui *et al.*, 1996), we have shown that the fractions B (IC$_{50}$ = 16.5 mg/

ml) and D (IC$_{50}$ = 15.6 mg/ml) on a DEAE-Sepha-dex A-25 column eluted with 0.1 and 0.3 M NaCl in 0.1 M phosphate buffer (pH 7.8), respectively, showed higher inhibitory activity against yeast AGH than the original hydrolyzate (IC$_{50}$ = 48.7 mg/ml). Thus, both fractions were subjected to the HPLC purification to isolate AGH inhibitory substrates.

Fig. 1 shows the elution patterns of fraction B on a TSK gel ODS-120T column, and the relative AGH inhibitory activity. The fraction was sepa-rated into two subfractions (elution volume of 12–18 ml and 20–24 ml, respectively). Most of the AGH inhibitory activity occurred in both subfrac-tions denoted as B-1 and B-2, respectively, though minor inhibition was also observed in other parts. The same purification was carried out for fraction D, from which the subfraction, D-1 (elution vol-ume of 67–71 ml under the same elution condi-tion) with potent AGH inhibition was collected (data not shown). These active fractions were used for the next purification step, and the HPLC run

monitored at 220 nm was performed on a Cos-mosil 5Ph column.

Fig. 2A shows the chromatogram of fraction B-2, and the active peak (arrow; retention time (RT); 17.9 min) was subjected to a final purifica-tion step on a Cosmosil 5C$_{18}$-AR II column (Fig. 2B). As shown a single peak with AGH in-

Fig. 2. HPLC purification of AGH inhibitory peptides from the active fraction of B-2 eluted on a TSK gel ODS-120T column. HPLC conditions of Fig. 2A: phenyl silica column (∅ 0.46×25 cm, Cosmosil 5Ph, Nacalai Tesque Co., Kyoto, Japan), eluted with a linear gradient of acetonitrile (5 to 50% for 45 min) at a flow rate of 0.5 ml/min. Subsequently HPLC (Fig. 2B) of the active fraction from a 5Ph column was performed with a C$_{18}$ column (∅ 0.46×25 cm, Cosmosil 5C$_{18}$-AR II, Nacalai Tesque Co., Kyoto, Japan), and elution with a linear gra-dient of acetonitrile (18 to 21% for 30 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. The elution was monitored at 220 nm.
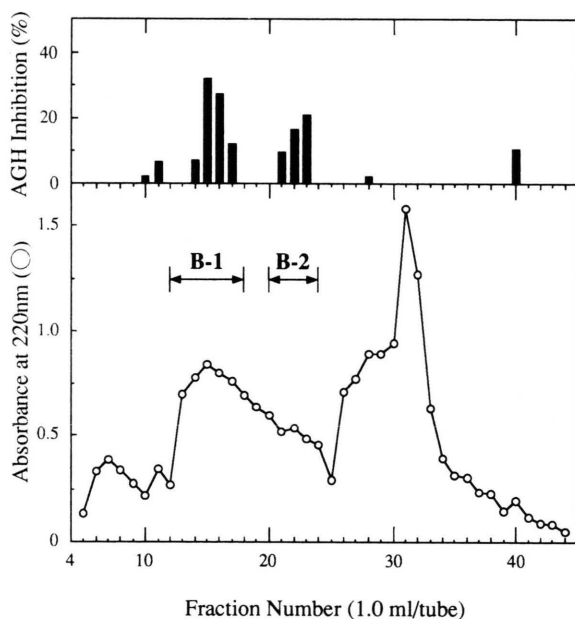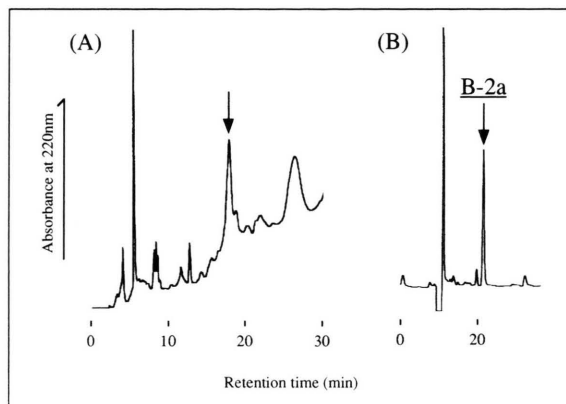

Fig. 1. Chromatography in a TSK gel ODS-120T column of active fraction eluted with 0.3 M NaCl from a DEAE-Sephadex A-25 column. The chromatographic condi-tions were as follows: column; TSK gel ODS 120T (∅ 0.78×30 cm), solvent system; (a) 5% CH$_3$CN (held for 10 min), (b) 50% CH$_3$CN (held for 15 min), linear gradi-ent from (a) to (b) in 45 min, flow rate; 1.0 ml/min, mon-itoring absorbance; 220 nm.

Table I. AGH inhibitory peptides isolated from sardine muscle hydrolyzate. The sequence of the isolated pep-tide was determined by both amino acid analyser and peptide sequencer.

Sequence	Amino acid ratio in acid hyrolyzate	IC$_{50}$[a] [mM]
Val–Trp	Val 1.00, Trp–	22.6 ± 0.36
Tyr–Tyr–Pro–Leu	Pro 1.00, Leu 1.05, Tyr 1.42	3.7 ± 0.02

[a] The inhibition ratio of each peptide against yeast α-glucosidase was evaluated by monitoring the pro-duction of PNP from PNP–G as a substrate at 400 nm after incubation at 37 °C for 15 min with or without inhibitor. The IC$_{50}$ values are expressed as mean ± standard deviation (n = 3).

hibitory activity denoted as B-2a was obtained (RT; 21.9 min). One peak denoted as D-1a (RT; 35.2 min) was also isolated from fraction D-1. Unfortunately, we could not isolate active peptides from B-1 fraction due to loss or disappearance of AGH inhibitory activity. As a result of amino acid composition and sequence analyses, all of the isolated AGH inhibitors were found for the first time in a natural protein hydrolyzate as a bioactive peptide, and identified as Val–Trp (B-2a; IC_{50} = 22.6 mM), and Tyr–Tyr–Pro–Leu (D-1a; IC_{50} = 3.7 mM) (Table I). Inhibitory activity of Tyr–Tyr–Pro–Leu was *ca.* 140-fold weaker than that of a voglibose, typical commercial therapeutic drug (Matsui *et al.*, 1996) (IC_{50} = 26 µM, Takeda Medical Co., Osaka, Japan). However, considering that the peptides may be used as a physiologically functional food component, the prophylactic effect in diabetes by food would be substantial, regardless of its lower inhibition. Fig. 3 shows the Lineweaver-Burk plots for the inhibition of AGH by both inhibitory peptides. As a result, both peptides inhibited AGH competitively. Both isolated peptides were constructed with not more than 4 amino acid residues, agreed with the result that the apparent molecular weight of the sardine muscle hydrolyzate was ranged from 250 to 1000 (Matsui *et al.*, 1993).

Importance of amino acid residue on the inhibition against AGH

In order to obtain the structural information of peptide required for the AGH inhibition, the relationship between the structure of derivatives from the isolated peptide and AGH inhibition was investigated (Table II). Tyr–Tyr–Pro–Leu which showed a potent inhibition against yeast AGH was selected as a skeleton peptide. As summarized five peptides were synthesized, and the AGH inhibition was determined. In comparison with the inhibitory activity of original tetra-peptide (IC_{50} = 3.7 mM), lack of Tyr at the N-terminus of the skeleton peptide did not influence on AGH inhibition power (IC_{50} = 3.9 mM), indicating that the tri-

Table II. AGH inhibitory activity of synthetic analogues of the peptide isolated from sardine muscle hydrolyzate. The analogue peptides were synthesized on the basis of the sequence of Tyr–Tyr–Pro–Leu isolated from the hydrolyzate.

Sequence	IC_{50}[a] [mM]
Tyr–Tyr–Pro–Leu	3.7 ± 0.02
Tyr–Pro–Leu	3.9 ± 0.02
Pro–Leu	n.i.[b]
Tyr–Pro	16.8 ± 0.08
Tyr–Pro–Gly	5.0 ± 0.01
Tyr–Pro–Tyr	25.8 ± 0.12

[a] The IC_{50} values are expressed as mean ± standard deviation (n = 3).
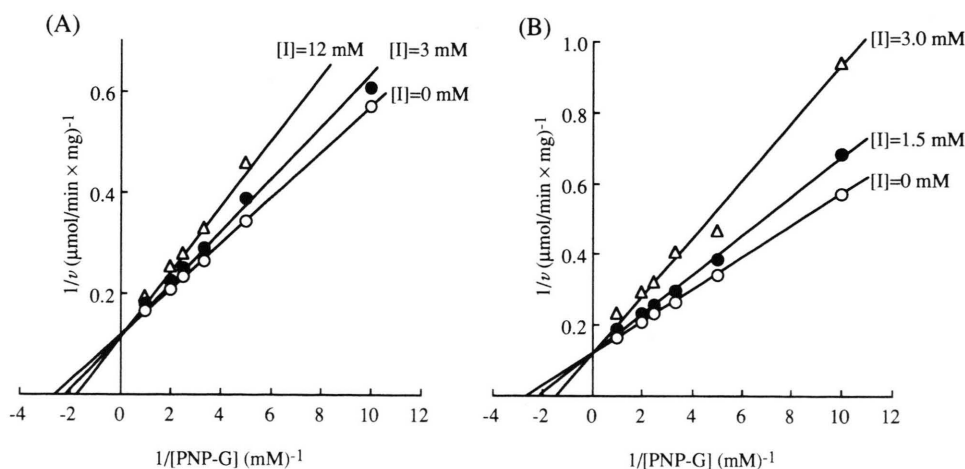[b] n.i., no inhibition under the assay conditions.

Fig. 3. Lineweaver-Burk plots for the inhibition of α-glucosidase (AGH) by Val–Trp (A) and Tyr–Tyr–Pro–Leu (B). The inhibition was measured by varying *p*-nitrophenyl α-D-glucopyranoside (PNP–G) concentration from 0.1 to 1.0 mM. The inhibitor concentrations are indicated on the linear regression lines.

structure, Tyr – Pro – Leu, was the essential element for AGH inhibition. In addition, the fact that the di-peptides, Pro – Leu, and Tyr – Pro as well as Val – Trp (Table I) showed no or less inhibition strongly suggests that tri-peptide chain length is required to decrease the action of AGH. Since Tyr – Pro showed poor inhibition, Leu at the C-terminus was found to be most important for binding to or interact with the active site of AGH. Substitution of Leu by Gly at the C-terminus of Tyr – Pro – Leu exhibited a similar AGH inhibition ($IC_{50} = 5.0$ mM), whereas a 6-fold reduction of inhibition activity was observed with Tyr – Pro – Tyr ($IC_{50} = 25.8$ mM). These results indicate that aliphatic amino acids, *e.g.* Leu or Gly at the C-terminus of the tri-peptide was more favorable than aromatic ones to inhibit AGH.

The aglycone of the pradimicin family of antibiotics potently inhibits AGH (Sawada *et al.*, 1993) as well as D-catechin (Nakahara *et al.*, 1993). So it appears that any AGH inhibitors should include a polyphenolic structure. This speculation was supported by Watanabe *et al.* (1993) and Nishioka *et al.* (1997), who isolated polyphenols and phenolic amides with potent AGH inhibitory activity from Tochu-cha and Welsh onion, respectively. Accordingly, a more effective amino acid at the N-terminus was assumed to be Tyr rather than an aliphatic amino acid. In addition, they have demonstrated that the existence of an amide proton available close to near the middle of the structure such as N-*p*-coumaroyltyramine is most effective to develop inhibitory activity (Nishioka *et al.*, 1997). Therefore, the amide proton from Pro at the middle position of the tri-peptide may be also important for a peptidic inhibitor to enhance its inhibition power, although the contribution of an amide proton to the inhibitory action against AGH has not yet been clarified.

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