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Original article

Inhibition of advanced glycation endproduct formation in amino acids mixture

Masayuki Yagi, Minaho Iida, Chieko Sakiyama, Yoshikazu Yonei

Anti-Aging Medical Research Center and Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

Accumulation of endogenous advanced glycation endproducts (AGEs) in the body due to glycative stress is one of the factors contributing to aging and the progression of age-related degenerative diseases. Methods to control glycative stress include controlling postprandial hyperglycemia, inhibiting AGE formation, and degrading and excreting AGEs. Since there are multiple pathways for glycation *in vivo*, in order to prevent AGE accumulation, it is necessary to inhibit the multifactorial pathways of the glycation. From these perspectives, a combination of materials is important for inhibition of AGE formation. Previous studies have shown that amino acids inhibit the formation of fluorescent AGEs (F-AGEs) and pentosidine. In this study, we prepared mixed solutions containing either two or six amino acids, selected seven amino acids known to have inhibitory effects on AGE formation, and examined their effectiveness based on their varying inhibitory effects on F-AGE and pentosidine formation. A model in which glucose was reacted with human serum albumin or type 1 collagen was used for the experiment. The results showed that the amino acid mixture had a higher inhibition rate of pentosidine formation than F-AGE formation. Ultimately, we identified to the combination with highest rate of inhibition for both F-AGE and pentosidine formation. This amino acid mixture may inhibit pentosidine formation, thereby reducing its cross-linking potentially preventing protein denaturation and dysfunction.

***KEY WORDS*:** glycation reaction inhibition, amino acid mixture, pentosidine, fluorescent AGEs

Introduction

The accumulation of advanced glycation endproducts (AGEs) in the body due to glycative stress is involved in the progression of aging and age-related degenerative diseases[1,2]. Glycation degrades protein function, causes inflammation when AGEs bind to RAGE (receptor for AGEs) expressed in immune cells, and leads to collagen cross-linking that reduces skin elasticity. Methods for suppressing glycative stress include controlling postprandial hyperglycemia, inhibiting the formation of AGEs, and decomposing and excreting AGEs[3]. There is growing interest in safer, food-derived functional ingredients that inhibit AGE production as alternatives to synthetic substances, such as aminoguanidine (AG), which has been associated with adverse events[4,5].

Ingredients that inhibit the AGE formation can be broadly divided into polyphenols, *i.e.*, catechins, anthocyanins[6,7] and proteins, peptides, amino acids. The former are found in tea, vegetables, and fruits[8-11], while the latter have been reported to be found in yogurt whey[12] and amino acids[13]. Glycation reactions in the body involve multiple pathways, leading to the production of a wide variety of AGEs[14]; therefore, it is desirable to inhibit multiple pathways to prevent AGE accumulation[15]. In practice, supplements that combine functional ingredients (*e.g.*, an extract made by mixing four types of herbs) are more likely to inhibit the AGE formation through multiple pathways[16], and clinical trials have shown high reproducibility in verifying the inhibitory effect of AGE formation[17-20].

From these perspectives, we hypothesized that a combination of materials is important for inhibiting AGE formation in the body. In this study, we used seven types of amino acids known to inhibit AGE formation, based

Contact Address: Visiting Professor Masayuki Yagi, PhD
Anti-Aging Medical Research Center / Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe-shi, Kyoto, 610-0394 Japan
TEL&FAX: +81-774-65-6394 e-mail: myagi@mail.doshisha.ac.jp
Co-authors: Iida M, ctuj2008@mail4.doshisha.ac.jp;
Sakiyama C, csakiyam@mail.doshisha.ac.jp; Yonei Y, yyonei@mail.doshisha.ac.jp

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on a previous report [13], and examined the effectiveness of combinations by comparing their inhibitory effects on fluorescent-AGE (F-AGE) and pentosidine formation in mixed solutions of two or six types of amino acids.

Materials and Methods

1) Reagents

The amino acids used in the experiment were provided by Ajinomoto Co., Inc. (Kawasaki, Kanagawa, Japan). Reagents were purchased from the following manufacturers. Human serum albumin (HSA, lyophilized powder, ≥ 96%, agarose gel electrophoresis) was purchased from Sigma-Aldrich Japan (Tokyo, Japan). Cowhide-derived collagen Type I (COL, derived from cowhide, pepsin-degraded) was purchased from Nippi (Tokyo). Aminoguanidine (aminoguanidine hydrochloride; AG) was purchased from Fujifilm Wako Pure Chemical Industries (Osaka, Japan). Other reagents were purchased as special grade or HPLC grade from Fujifilm Wako Pure Chemical Industries or Nacalai Tesque (Kyoto, Japan).

2) Samples

The following seven types of samples were used from among amino acids that have been reported to have inhibitory effects on the F-AGE and pentosidine formation [13]. Sodium aspartic acid hydrate (Asp), histidine hydrochloride (His), threonine (Thr), cysteine hydrochloride hydrate (Cys), cystine (Cys-Cys), glycine (Gly), and ornithine monohydrochloride (Orn). The amino acids were dissolved and diluted in water or ethanol solution before use as sample solutions. The total amino acid concentration of the sample solution was 10 mmol/L, and an equal mixture of 10 mmol/L solutions was prepared for single amino acids, 5 mmol/L solutions for two amino acid mixtures, and 1.7 mmol/L solutions for six amino acid mixtures.

3) Protein-glucose glycation reaction model

To verify the inhibitory effect on glycation, a protein-glucose glycation model was used with reference to a previous report [21]. The amino acid sample was mixed with 0.1 mol/L phosphate buffer (pH 7.4) containing protein and glucose to prepare a reaction solution. The reaction solution composition of the glycation model was HSA 8 mg/mL and glucose 0.2 mol/L (HSA-model), COL 1.2 mg/mL and glucose 0.4 mol/L (COL-model).

For the glycation reaction, a solution containing all of the phosphate buffer, protein solution, glucose solution, and sample solution (A), a solution containing purified water instead of the glucose solution in A (B), a solution containing sample dissolving solution instead of the sample solution in A (C), and a solution containing purified water instead of the glucose solution in C (D) were prepared and incubated at 60 °C. The incubation time was 40 hours for the HSA-model and 10 days for the COL-model.

4) Measurement of AGEs

F-AGEs were measured as previously reported [21] by centrifugal filtration of the glycation solution after incubation using a 30K ultrafiltration membrane unit (Merck, Datmstadt, Germany), redissolving the protein fraction with a molecular weight of 30K or more in 0.1 mol/L phosphate buffer (pH 7.4), and then 200 µL was placed in a black microplate to measure AGE-derived fluorescence (excitation wavelength 370 nm / fluorescence wavelength 440 nm). Pentosidine was measured as previously reported [22] by mixing 50 µL of the glycation reaction solution with 6 N hydrochloric acid, hydrolyzing at 105 °C for 18 hours, and then measuring by HPLC.

5) Calculation of the AGE formation inhibition rate

The AGE formation inhibition rate was calculated by the following formula for the F-AGE and pentosidine formation inhibition rate (%), as previously reported [21]. AG was used as the positive control for AGE formation inhibition.

$$\text{Inhibition rate (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

Statistical analysis

Measurements are shown as the average of duplicate measurements, and t-tests were used to compare between the two groups. Spearman's rank correlation coefficient was used to analyze correlation. Statistical analysis results were considered significant at a risk level of less than 5%.

Results

Inhibitory effect of two amino acid mixtures on the F-AGE and pentosidine formation

The samples examined were 20 pairs of two-amino-acid mixtures (equal mixtures of 5 mmol/L solutions) and seven types of single amino acids (10 mmol/L) (***Table 1***). The AGE formation inhibition rate in the HSA-model for two-amino-acid mixtures was higher for F-AGEs (38.2 ± 22.6%, n = 20) and pentosidine (73.4 ± 13.4%, n = 20) than for F-AGEs ($p < 0.001$). The pentosidine formation inhibition rate was more than twice as high as the F-AGE formation inhibition rate in nine of the pairs. Similarly, in the COL-model, the AGE formation inhibition rates for F-AGEs (36.7 ± 22.0%, n = 20) and pentosidine (67.3 ± 13.7%, n = 20) were higher than those for F-AGEs ($p < 0.001$). Furthermore, in the COL-model, the pentosidine formation inhibition rate was more than twice as high as the F-AGE inhibition rate for four pairs of two-amino-acid mixtures.

The F-AGE formation inhibition effect was more than 15% higher than the average formation inhibition rate of single amino acids for mixtures of Thr and Gly, and Thr and Asp in the HSA-model, and for mixtures of Cys-Cys and Gly, Cys-Cys and Thr, and Cys-Cys and Asp in the COL-model. Similarly, the inhibitory effect on pentosidine formation was 15% or more higher than the average inhibition rate of single amino acids when combining His and Gly in the HSA model, and when combining Thr and Asp, and Thr and Orn in the COL model. A correlation was observed between the F-AGE and pentosidine formation for the two-amino-acid mixtures in the HSA model ($p < 0.001$). However, no similar correlation was observed in the COL model. A correlation was observed in the F-AGE inhibition rate for the two-amino-acid mixtures between the

HSA- and COL-model ($p = 0.021$). No similar correlation was observed for the inhibition rate of pentosidine formation.

Inhibitory effect of six amino acid mixtures on the F-AGE and pentosidine formation

The six amino acid mixtures examined (equal mixtures of 1.7 mmol/L solutions) were: a mixture containing five amino acids, His, Gly, Asp, Orn, and Thr, plus Cys-Cys (Combination A), and a mixture containing Cys instead of Cys-Cys (Combination B) (***Table 2***). The AGE formation inhibition rate of the six amino acid mixtures was 1.4 times higher for F-AGEs and 1.2 times higher for pentosidine in the HSA-model than in Combination A and B. Conversely, the AGE formation inhibition rate of the COL-model was 0.9 times higher for F-AGEs and 1.0 times higher for pentosidine, which were equivalent.

Table 1. Inhibition rate (%) of AGEs formation by two amino acids mixture.

amino acids mixture		HSA (%)		COL (%)	
		F-AGEs	Pentosidine	F-AGEs	Pentosidine
Cys-Cys	His	58.6	91.8	30.9	90.7
Cys-Cys	Gly	66.8	87.3	75.7	83.0
Cys-Cys	Thr	66.1	89.1	75.1	82.3
Cys-Cys	Asp	66.0	85.4	84.2	83.4
Cys-Cys	Orn	61.3	84.2	52.9	80.1
His	Gly	26.4	70.9	ND	65.9
His	Cys	55.7	88.0	1.8	68.7
His	Thr	27.8	69.4	ND	75.0
His	Asp	23.4	65.7	ND	57.8
His	Orn	22.1	79.0	ND	58.4
Gly	Cys	59.4	81.0	64.1	63.8
Gly	Thr	19.9	55.2	48.9	71.5
Gly	Asp	27.8	54.3	39.5	57.6
Gly	Orn	ND	55.6	ND	59.2
Cys	Thr	52.2	78.2	61.6	55.3
Cys	Asp	54.7	79.2	65.9	45.9
Cys	Orn	52.7	83.1	17.7	36.5
Thr	Asp	11.9	46.4	60.5	70.5
Thr	Orn	5.8	61.5	48.3	82.8
Asp	Orn	5.4	62.5	6.3	57.2
Cys-Cys		100.2	101.6	75.9	87.2
His		11.3	64.0	ND	69.5
Gly		12.7	43.5	36.2	68.1
Cys		77.1	94.4	66.3	60.5
Thr		34.5	51.2	30.0	56.7
Asp		29.7	44.4	48.6	52.7
Orn		6.4	69.9	ND	51.3

Data; mean (n = 2), ND; not effective, HSA; human serum albumin-glucose glycation model, COL; collagen-glucose glycation model, F-AGEs; fluorescent AGEs

Table 2. Inhibition rate (%) of AGEs formation by six amino acids mixture.

amino acids mixture	HSA (%)		COL (%)	
	F-AGEs	Pentosidine	F-AGEs	Pentosidine
His, Gly, Asp, Orn, Thr, Cys-Cys	49.1	81.6	57.9	82.9
His, Gly, Asp, Orn, Thr, Cys	35.1	67.2	62.3	85.5

Data; mean (n = 2), ND; not effective, HSA; human serum albumin-glucose glycation model, COL; collagen-glucose glycation model, F-AGEs; fluorescent AGEs.

Discussion

Inhibitory effect of amino acid mixture on the AGE formation

The mechanism by which amino acids inhibit AGE formation is assumed to involve blocking the AGE formation pathway by preventing the formation of Amadori compounds, which are the initial stage and intermediates of glycation reactions[13]. The inhibitory effect of amino acids on AGE formation differs depending on the type of amino acid. Consequently, the inhibition of AGE formation by amino acid mixtures may affect multiple pathways and inhibit the formation of multiple types of AGEs, depending on the type of amino acid combined. It has already been reported that a mixed herb extract of *Houttuynia cordata*, chamomile, hawthorn, and grape leaves inhibits multiple pathways of AGE formation[16] and reduces AGE formation in the blood and skin in clinical intake tests[17-20]. Additionally, the inhibitory effect of the two-amino-acids mixtures on F-AGE formation was found to be 15% or more higher than the average inhibition rate of the formation of each single amino acid depending on the type of amino acid mixed, Thr in the HSA-model and Cys-Cys in the COL-model. A similar effect was observed for Thr in the COL-model regarding its inhibitory effect on pentosidine formation, and for His or Gly in the HSA-model. The change in inhibitory effect on the AGE formation by mixing amino acids suggests that, similar to the mixed herb extract, the combined amino acids may have a complementary effect on inhibiting the F-AGE formation.

The AGE formation inhibitory effect of amino acid mixture in HAS- and COL-model

The AGE formation inhibitory effect of amino acid mixture showed differences in the inhibition rate in HSA and COL models, which were used as glycation model proteins in this study, and the inhibition rates of the F-AGE and pentosidine formation. The F-AGE formation inhibitory effect of 41 types of spices was compared by changing the combination of proteins (HSA, BSA, COL) used as glycation models, and no correlation was found in the IC_{50} values among the protein types[23]. However, a correlation was found in the IC_{50} values when the type of sugar (glucose, fructose) reacting with the glycation model protein was changed. When the F-AGE formation inhibitory effect was compared using a complex mix with four types of plant extracts or AG as a sample, the HSA-model, COL-model, and keratin model proteins were used, and the F-AGE formation inhibitory effects differed[24]. It is believed that the AGE formation inhibitory effect of spices and plant extracts is due to polyphenols contained in the raw plants. In this study, a correlation was observed in the F-AGE formation inhibition rate of the amino acid mixture between the HSA and COL model. A correlation was observed only for HSA, but not for COL, between the F-AGE and pentosidine formation inhibition rate of amino acids. The AGE formation inhibition effect of amino acids varies depending on the model protein and the type of AGEs, and the AGE formation inhibition effect differs from that of polyphenols. The sites of AGE formation in proteins are the N-terminal amino acid, and the arginine and lysine residues in the protein molecule. In addition, the hydrophilicity and hydrophobicity around the arginine and lysine residues in the protein molecule differ depending on the amino acid sequence and three-dimensional structure[25,26]. Since each amino acid has characteristics such as hydrophilicity, hydrophobicity, acidity, basicity, and neutrality, the effect of amino acids on the glycation site of a protein varies depending on the amino acid type. The difference in the AGE formation inhibition effect of amino acid mixtures observed suggest the possibility that the amino acid mixture acts complementarily on the model proteins.

The usefulness of amino acids in inhibiting the AGE formation

Amino acids are components found in various foods, serve as bulding block for synthesizing biological proteins, and have functions such as anti-oxidants and anti-glycation properties[27]. They also contribute to the taste and umami of foods[28]. Food rich in amino acids include fermented foods like meat and seafood soups, miso, soy sauce, and cheese, and are used in cuisines worldwide[29-30]. The amino acids contained in these foods are produced by the decomposition and aging of animal or plant proteins used as ingredients. For this reason, fermented foods have different amino acid compositions and amounts depending on the type of protein used as a raw material, which affects their taste and functionality[31]. However, it is said that there is no shortage of amino acids in everyday meals[32]. If even one essential amino acid is lacking, it cannot be effectively used in the body, making it is important to have a balance in intake (satisfying the amino acid score). Although amino acids alone have an inhibitory effect on the AGE formation, they may act complementary when two or more types are mixed. From this perspective, foods containing multiple amino acids are of superior quality and may help inhibit glycation after ingestion. Furthermore, the inhibitory effect of the amino acid mixture on the AGE formation was stronger for pentosidine, a cross-linked AGE, than for F-AGEs, suggesting that this inhibition may help prevent protein hardening and denaturation. It has been reported that protein hardening due to glycation progresses with age in skin and bone collagen[33-35]. The results of this study suggest that ingesting a combination of amino acids that are easy to incorporate into our everyday diet may be useful in inhibiting glycative stress.

Research limitations

Amino acids are components found in all foods and have been reported to have various functions in the body. The AGE formation inhibitory effect of the amino acid combination in this study was determined through an *in vitro* test. The usefulness of the AGE formation inhibitory effect of amino acids on the body needs to be verified in human clinical trials.

Conclusion

AGE formation inhibition was verified for the HSA and COL model using a mixture of two or six amino acids as a

sample. The effect of the amino acid mixture was higher than the F-AGE formation inhibition rate. Eventually, we identified the combination in which the F-AGE and pentosidine formation inhibition rate of the amino acid mixture were the highest. This amino acid mixture may inhibit pentosidine formation, reducing its cross-linking potential and potentially preventing protein denaturation and dysfunction.

Conflict of interest declaration

Ajinomoto Co., Inc. provided amino acid samples and research funds for this study.

Acquisition of competitive funds

None in particular

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