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Regulatory Effect of Amino Acids on the Pasting Behavior of Potato Starch Is Attributable to Its Binding to the Starch Chain

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The binding of an amino acid, glycine (Gly), alanine (Ala), ϵ-aminocaproic acid (ϵ-AC), monosodium glutamate (GluNa), or lysine (Lys), to starch was examined by a biomolecular interaction analyzer (IAsys). A starch sample (ATS) hydrolyzed to an extent of 1% hydrolysis rate with 15% sulfuric acid was used as a model starch for the binding examination. The reducing end of ATS was oxidized by the Somogyi reagent, and the conversion of the reducing end to the carboxyl group of ATS was confirmed by a carboxylic acid fluorescence labeling reagent. The oxidized ATS was immobilized to the amino group of a sensor cuvette by using water-soluble carbodiimide and *N*-hydroxysuccinimide through an amide bond. The IAsys examination showed that Gly, Ala, and ϵ-AC scarcely bound to the immobilized starch chains but that GluNa and Lys favorably bound with their increasing concentrations. The relative binding index (RBI) of each amino acid was defined by the ratio of the slope of the linear regression equation between the binding response and the concentration for each amino acid to that for Gly. Because the relationships between the RBI and the pasting characteristics (pasting temperature, peak viscosity, breakdown, and swelling index) could each be expressed by a linear regression equation with a high correlation coefficient, it is concluded that the regulation of the pasting behavior of starch with an amino acid is caused by binding of the amino acid to the starch chains.

KEYWORDS: Amino acids; potato starch; starch pasting

INTRODUCTION

Starch is an essential energy source and the main component of crops such as corn, wheat, rice, potato, sweet potato, cassava, and sago. Starch is applied to many processed foods as a builder or modifier of their physical properties and structures. Starch occurs in nature as insoluble granules. Heating starch granules in water causes them to absorb a large quantity of water and to extensively swell, which is referred to as gelatinization, and results in revealing these functional properties. However, the swollen starch granules can easily break down under a shearing stress, resulting in the development of a sticky and pasty texture that is often disliked in starchy foods. It thus becomes important to regulate the swelling of the starch granules and the viscosity of the gelatinized starch suspension. It is well-known that such coexisting substances as salts (*1*−*6*), sugars (*1*, *6*, *7*), alcohols (*8*, *9*), fatty acids (*10*), surfactants (*6*, *11*), organic acids (*9*), and amino acids (*12*−*14*) influence the gelatinization behavior of starch in terms of changes in the gelatinization temperature, crystallinity, viscosity, swelling, digestibility, and transparency. It is therefore necessary to choose the most suitable coexisting substance to regulate the gelatinization behavior of starch that will achieve the required physical properties. We paid attention to amino acids, which have previously received little attention, and clarified that amino acids and a peptide such as ϵ-poly(L-lysine) (PL) elevated the gelatinization temperature and reduced the viscosity and swelling of potato starch. In particular, lysine (Lys), monosodium glutamate (GluNa), and PL with a positive or negative net charge each had a strong influence on increasing the gelatinization temperature and decreasing the viscosity and swelling, whereas glycine (Gly) and alanine (Ala) with a zero net charge had little effect (*13*), and these effects strongly depended on the absolute value of the amount of their net charge (*14*). Ling and King have also reported that charged amino acids showed stronger influence on rice starch pasting than neutral ones (*12*). It is thus considered that such an influence of amino acid is not peculiar to potato starch. However, the mechanism for the particular regulatory effect of amino acids has not previously been clarified, while the reason for the effects of coexisting substances like salts on the gelatinization behavior has not been interpreted beyond their relationship with the lyotropic effect (*4*), hydration number of ions (*1*), and *B*-coefficient (*1*) of the viscosity equation of a salt solution

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Table 1. Reducing End Contents of ATS and Oxidized ATS (Ox-ATS)

content	ATS	Ox-ATS
relative reducing end content to total sugar content (%)	1.282	0.3057
relative value of Ox-ATS to ATS (%)	100.0	23.9
oxidation ratio (%)	0.0	76.1

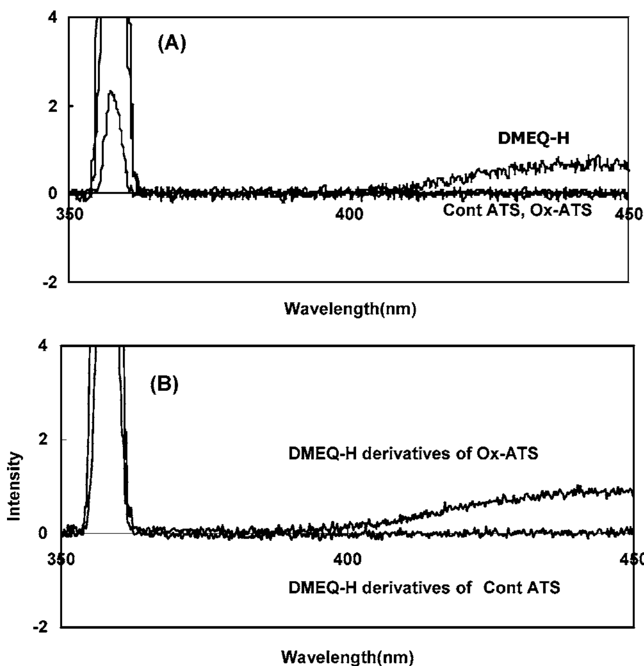

Figure 1. Fluorescent spectra of the DMEQ-H derivatives of Ox-ATS and ATS (**B**) as compared with those of DMEQ-H, Cont ATS, and Ox-ATS (**A**).

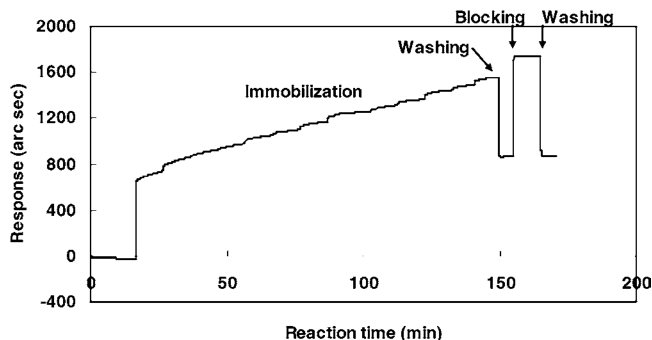

Figure 2. IAsys response during the immobilization of Ox-ATS to an amino cuvette.

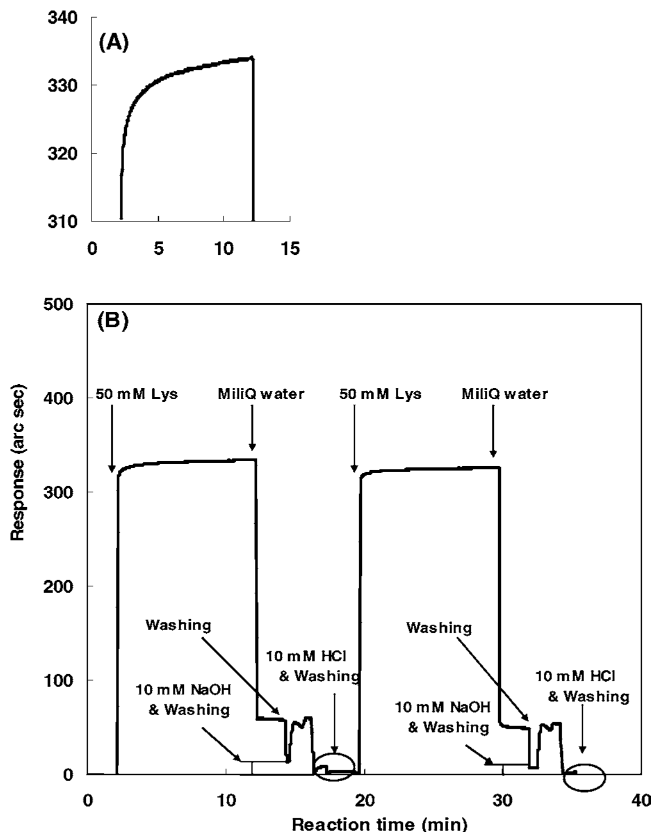

Figure 3. Binding and dissociation profiles after adding 50 mM Lys and then replacing with distilled water.

representing the strength of interaction between the salt ion and the solvent. It is therefore necessary to experimentally demonstrate starch binding to elucidate the interaction among water, starch, and coexisting substances.

A biomolecular interaction analyzer can be used for analyzing in real time the binding behavior of various biomolecules by detecting the binding signal depending on the mass of the ligand bound to the acceptor molecule of the immobilized resonant layer of a sensor cuvette (*15*−*19*). In this study, the biomolecular interaction analyzer is applied to demonstrate the binding behavior of amino acids to starch chains by preparing a starch chains-immobilized sensor cuvette, and we show how such binding behavior regulates the pasting characteristics of potato starch granules.

MATERIALS AND METHODS

Materials. Potato starch (Hokuren Research Institute, Sapporo, Japan) was used after being repeatedly washed with distilled water at 4 °C and air-dried (13.7% moisture). Gly, Ala, GluNa, and Lys were obtained from Ajinomoto Co. (Tokyo, Japan). 6,7-Dimethoxy-1-methyl-2(H)-quinoalinone-3-propinohydrazide (DMEQ-H), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ϵ-amino caproic acid (ϵ-AC) were purchased from Wako Pure Chemical Co. (Osaka, Japan). All other reagents used were commercially available.

Preparation of the Acid-Treated Potato Starch (ATS). ATS was prepared according to the previously reported method (*20*). In brief, potato starch was immersed in 15% sulfuric acid at 25 °C and gently stirred two or three times a day until the amount of dissolved saccharides reached to 1%. The sulfuric acid was removed with a G-4 glass filter. The resulting starch granules were thoroughly washed with distilled water until the pH value of the filtrate reached that of distilled water before being dissolved in distilled water by heating at 80 °C for 5 min, dialyzed against distilled water to eliminate the maltooligosaccarides, and finally lyophilized to recover ATS. The average degree of polymerization of ATS was evaluated to be 78.0 from the ratio of the total sugar content to reducing sugar content.

Preparation of the Reducing End-Oxidized Starch (Ox-ATS). ATS (400 mg) was dissolved in 40 mL of distilled water by heating at 80 °C for 5 min. The ATS solution (2 mL) and 2 mL of the Somogyi reagent (*21*) were mixed. After they were heated in boiling water for 1 h, the contents were cooled to 20 °C and centrifuged at about 900*g* for 5 min at 20 °C. The resulting supernatant was adjusted to pH 2.0 with 4 M HCl, thoroughly dialyzed against distilled water, and finally lyophilized to recover Ox-ATS. A similarly treated starch sample without being heated was also prepared as the control (Cont ATS). The degree of oxidization of ATS was evaluated from the ratio of the reducing end content to the total sugar content before and after oxidation, respectively, determined by the Somogyi (*21*)−Nelson method (*22*) and the phenol−sulfuric acid method (*23*).

Fluorometry of the Carboxyl Group End of Ox-ATS. Cont ATS or Ox-ATS (10 mg) was dissolved in 500 μL of distilled water by

Figure 4. Binding profiles evaluated by IAsys after adding Gly, Ala, ϵ-AC, GluNa, and Lys solutions at 50, 100, 200, 400, and 600 mM evaluated by IAsys.

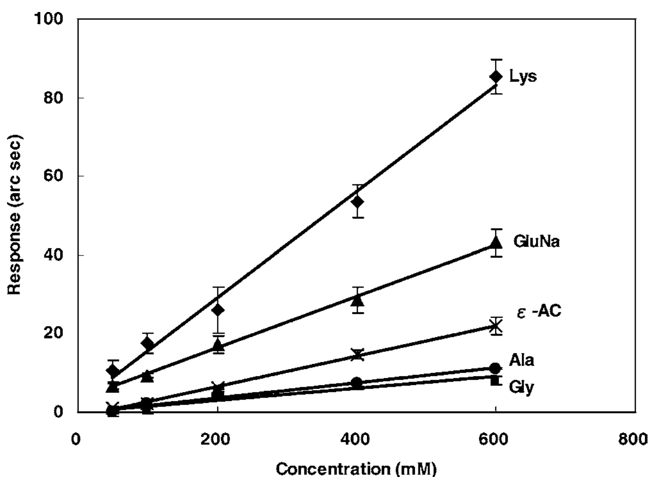

Figure 5. Binding of Lys (♦), GluNa (▲), ϵ-AC (×), Ala (●), and Gly (■) to the starch chains.

heating at 80 °C for 5 min. After 50 μL of 50 mM DMEQ-H-dimethylformamide solution and 50 μL of 2 M EDC to 120 μL of the Cont ATS or Ox-ATS solution were added, the reaction mixture was heated at 37 °C for 5 min. The unreacted reagent was removed by dialyzing against distilled water. The fluorescence spectrum of the sample solution was measured under excitation at 360 nm by using an RF-5300PC fluorospectrophotometer (Shimadzu, Kyoto, Japan).

Immobilization of Ox-ATS to an Amino-Cuvette of IAsys. An Ox-ATS-immobilized cuvette was prepared by using an amino cuvette with amino groups as the resonant layer for evaluating the binding of amino acids to the starch chain by an IAsys biomolecular interaction analyzer (Affinity Sensors, Cambridge, United Kingdom) as follows. The amino cuvette was washed with 10 mM phosphate-buffered saline (PBS) at pH 7.0. After an activating reaction solution containing 120 μL of 0.4 M EDC and 120 μL of 0.1 M NHS was prepared, the activating solution and the Ox-ATS solution (200 μg/mL) dissolved in 10 mM PBS were mixed at 1:1 (v/v) and left at room temperature for 5 min to activate the carboxyl groups of Ox-ATS. This reaction solution (200 μL) was loaded into the amino cuvette and then reacted for 10 min to immobilize Ox-ATS to the amino groups of the cuvette through an amide bond. The immobilization process was repeated several times until the signal response reached a plateau. To block the unreacted amino groups of the cuvette, 200 μL of 0.2 M sodium acetate that had been activated with EDC/NHS as just described was added and then reacted for 10 min. After they were washed 10 times with PBS, starch-immobilized cuvettes with 387.0, 448.3, 887.6, and 1583.7 arc sec signal responses were obtained. These cuvettes were filled with PBS and kept in a refrigerator until used.

Evaluation by IAsys of Amino Acids Binding to the Starch Chain. The binding of amino acids to the starch chain was measured with an IAsys plus biomolecular interaction analyzer (Affinity Sensors Co.) as previously described (*15*, *16*). PBS in the starch-immobilized cuvette was replaced with MilliQ water adjusted to pH 7.0 with 10 mM NaOH, washed 10 times with 10 mM NaOH, and then completely replaced with MilliQ water (pH 7.0). A Lys, Glu, Gly, Ala, or ϵ-AC solution (200 μL) at a concentration of 50, 100, 200, 400, or 600 mM was added to the cuvette, and the binding with the immobilized starch chains was monitored by the resonant angle of the laser light at 670 nm. After the binding response had been measured for 10 min, the sample was dissociated by replacing the medium with MilliQ water (pH 7.0) for 2 min, and the cuvette was regenerated by washing 10 times with 10 mM NaOH, washing three times with MilliQ water, replacing with 10 mM HCl for 1 min, and washing three times with MilliQ water until the response had recovered to the initial value.

Table 2. RBI[a] of Gly, Ala, ε-AC, GluNa, and Lys to the Starch Chain and Linear Regression Equations Obtained from RBI (X) and the Pasting Characteristics (Y)[b] Evaluated by Rapid Viscoanalyzer (RVA) and Swelling Measurements

	Gly	Ala	ε-AC	GluNa	Lys
RBI (*X*)	1.00	1.07	1.47	2.22	4.59
		pasting characteristics (*Y*)			
pasting temp (PT; °C)	70.9	71.0	71.0	81.9	81.8
peak viscosity (PV; RVU)	581.4	542.5	580.9	225.2	230.4
breakdown (BD; RVU)	432.6	392.5	426.5	97.5	103.8
swelling index (SI)	32.1	32.8	34.0	18.3	18.0

relationship	linear regression equation	correlation coefficient (*r*)
PT (*Y*)/RBI (*X*)	$Y = 3.26X + 68.6$	0.815
PV (*Y*)/RBI (*X*)	$Y = -101.2X + 641.6$	0.806
BD (*Y*)/RBI (*X*)	$Y = -94.1X + 485.3$	0.805
SI (*Y*)/RBI (*X*)	$Y = -4.44X + 36.2$	0.813

[a] Relative ratio of the slope of the linear regression equation for each amino acid obtained from **Figure 5** to that for Gly. [b] RVA and swelling measurements were carried out after adding 2% w/w of amino acids based on the dry starch weight (*14*).

RESULTS AND DISCUSSION

Confirmation of a Carboxyl Group at the Terminal of Ox-ATS and Immobilization to the IAsys Cuvette. The reducing end of the starch chain was oxidized by the Somogyi reagent to immobilize ATS to the amino groups on the resonant layer of the amino cuvette of a biomolecular interaction analyzer (IAsys) through an amide bond. An ATS concentration of 10 mg/mL and a reaction time of 60 min with the Somogyi reagent were selected from the results of a preliminary examination of the optimum oxidation conditions. Because the unreacted Somogyi reagent could not be removed by dialyzing against distilled water in the alkaline pH range, the reaction solution after removing the precipitate of copper oxide was adjusted to pH 2.0, dialyzed against distilled water, and lyophilized to obtain Ox-ATS. The yield of Ox-ATS was 82.5%, and the oxidization ratio was evaluated to be 76.1% from the relative reducing end content of OX-ATS (23.9%) (**Table 1**). H NMR measurement of Ox-ATS could not provide distinct evidence for the chemical shift of the proton of the carboxyl group appearing at 10−15 ppm due to the very low concentration of the target proton, so the carboxyl group was confirmed by fluorescence-labeling Ox-ATS. DMEQ-H having a hydrazide group as an active group was selected as a fluorescent reagent for labeling the carboxyl group in this study, because it is highly sensitive, soluble in an aqueous solution, and enables a reaction at room temperature (*24*). DMEQ-H showed clear fluorescence in a wavelength region of 410−450 nm (**Figure 1A**), while Ox-ATS and Cont ATS showed no fluorescence. The Ox-ATS sample labeled with DMEQ-H showed clear fluorescence at 410−450 nm (**Figure 1B**), whereas the Cont ATS sample treated with DMEQ-H showed no fluorescence. These results confirmed that Ox-ATS had a carboxyl group at the terminal end of the starch chain.

Ox-ATS was immobilized to an amino cuvette of IAsys by the mediation with EDC and NHS. The carboxyl group of Ox-ATS that had been activated by EDC and NHS was combined with the amino groups of the amino cuvette through an amide bond. When the Ox-ATS and the EDC/NHS mixture (1:1) were added to the amino cuvette, the response signal of IAsys occurred without any delay, indicating immobilization of Ox-ATS to the amino groups. This response continued to increase for 10 min, so the immobilization procedure was conducted several times to immobilize a sufficient amount of Ox-ATS. The unreacted amino groups were blocked by adding sodium acetate and the EDC/NHS mixture (1:1) to the cuvette, resulting in a further increase in the response, which soon reached a plateau (**Figure 2**). It is thus considered that the remaining amino groups had been entirely blocked. Four kinds of ATS-immobilized cuvette with responses of 387.0, 448.3, 887.6, and 1583.7 arc sec could be prepared.

Regulation of Gelatinization by Binding Amino Acids to the Starch Chain. IAsys was applied to investigate amino acids binding to the starch chain by using the ATS-immobilized cuvette. When a 50 mM Lys solution was added to the cuvette with the response of 887.6 arc sec, an immediate response signal occurred and then gradually increased after an initial sudden increase being a bulk shift due to replacing the medium from MiliQ water to the Lys solution (**Figure 3**). This gradual increase is believed to have indicated the binding progress of Lys to the starch chains and presents the first evidence for binding of amino acids to starch chains. After measuring the binding response, the medium was replaced with MilliQ water in an attempt to dissociate the bound Lys. However, adequate dissociation did not probably occur due to rebinding of the dissociated Lys, preventing the dissociation rate constant (K_{diss}) and dissociation constant (K_D) from being obtained. Therefore, 10 mM NaOH and 10 mM HCl were used as a regeneration medium, resulting in recovery to the initial response value. Then, readding the Lys solution gave a reproducible binding curve (**Figure 3**). It was therefore considered that the binding behavior of amino acids to starch chains could be evaluated by using the Ox-ATS-immobilized cuvette. Of the four prepared cuvettes, the two with responses of 387.0 and 448.0 arc sec did not show an adequate response to the addition of an amino acid due to the small amount of the immobilized Ox-ATS. The cuvette with the response of 1583.7 arc sec indicated poor response probably due to too large immobilization of Ox-ATS that resulted in occupation of the evanescent field where the place generating the resonance is depending on the binding of Lys. The cuvette with the response of 887.6 arc sec was therefore used to analyze the binding behavior of the amino acids.

The binding behavior was analyzed for Gly, Ala, ε-AC, GluNa, and Lys solutions. The binding response of Lys and GluNa increased with the increasing concentration, whereas Gly, Ala, and ε-AC showed only a small increase (**Figure 4**). The binding ability to starch chains therefore differed with the type of amino acid. The detected response was corrected in this study by dividing it by the molecular weight of each amino acid, because the response depends on the molecular weight. Because an increased response indicates the degree of binding ability of amino acids to starch chains, the increased response after 10

min until the plateau was reached was plotted against the sample concentration (**Figure 5**); Lys and GluNa again demonstrated an elevated response with increasing concentration, whereas Gly and Ala showed hardly any increased response. There was a linear relationship with a high correlation coefficient for each amino acid. It was thus considered that the degree of the binding ability of amino acids to starch chains could be concisely expressed by the slope of the linear regression curve. However, the linear regression curve showed a small scatter with measuring day (data not shown), because the reactivity of the immobilized starch chains to amino acids could have fluctuated depending on the extent of reconstruction of the ordered structure, a kind of retrogradation, during the time that the cuvette was stored in the refrigerator. The binding ability of each amino acid is thus defined in this study as the relative binding index (RBI) by the ratio of the slope for each amino acid to that for Gly (**Table 2**).

The previous study (*15*) had shown that amino acid could regulate the gelatinization temperature, pasting viscosity, and swelling of potato starch granules depending on the absolute value of its net charge, probably due to electrostatic interaction. The pasting characteristics and swelling index measured in that study after adding of 2% w/w of amino acids based on the dry starch weight were applied to investigate the relationship between the RBI and the effects of amino acids on the pasting behavior. These parameters increased with increasing RBI, and the relationships between the pasting parameters (pasting temperature, peak viscosity, breakdown, and swelling index) and the RBI could be expressed by each linear regression equation with a high correlation coefficient of above 0.8 (**Table 2**). These findings indicate that amino acids with good binding ability could effectively inhibit the swelling of potato starch granules. Because it is considered that the volume of a swollen starch granule is influenced by the balance between the rigidity of the hydrogen bond among those starch chains that maintain a limited granular size and the force created by granule expansion due to the influx of water, that is, by competition between the structural factors of the starch granules and the solvating ability of the solution, those substances with suitable binding ability to starch such as Lys and GluNa are thought to effectively reduce swelling of the starch granules by inhibiting the interaction between the starch and the solvent due to binding to starch chains. This results in an increased pasting temperature and decreased peak viscosity, breakdown, and swelling index. It therefore seems that the pasting behavior of potato starch granules can be according to various demands regulated by selecting amino acids with appropriate binding ability to the starch chains.

In conclusion, a biomolecular interaction analyzer (IAsys) was applied to prove the binding of amino acids (Gly, Ala, ϵ-AC, GluNa, or Lys) to starch chains. The reducing end of ATS with a 1% hydrolysis ratio was oxidized by the Somogyi reagent to be immobilized to an amino cuvette of IAsys through an amide bond. IAsys measurements with this oxidized ATS-immobilized cuvette exhibited poor binding with Gly, Ala, and ϵ-AC but favorable binding with GluNa and Lys. The relationships between the defined RBI of each amino acid to the starch chains and the pasting temperature, peak viscosity, breakdown, and swelling index of the starch granules indicated a high correlation coefficient for each. It is thus concluded that the regulation of the pasting behavior of starch with amino acids is caused by binding of the amino acids to starch chains.

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