Master's Thesis of Science in Agricultural Biotechnology

Development of label-free colorimetric aptasensor based on the aggregation of gold nanoparticles for detection of gliadin in real foods

실제 식품에서 글리아딘 검출을 위한 금 나노입자 응집 기반 라벨프리 비색 압타센서 개발

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ABSTRACT

Gluten, composed of glutenin and gliadin, is an important raw material that determines the quality of wheat foods such as bread and pasta by giving dough viscoelasticity. However, gliadin in the gluten protein causes celiac disease and wheat allergens, which causes fatal damage to the small intestine, so many methods have been developed for the detection of gluten. The studied detection method has disadvantages that are difficult to use practically in the field due to complicated equipment, long time, and cost. In this paper, a label-free colorimetric aptasensor detection method that can detect gliadin in gluten, a cause of celiac disease, was developed in the field. Based on the salt-induced aggregation of gold nanoparticles, it was detected using a gli4 aptamer that specifically recognizes gliadin. The pre-treatment process of gliadin was simplified to be suitable for the field by extracting it by dissolving it in 60% ethanol. For induction of salt aggregation of gold nanoparticles, gli4 aptamer, gliadin, and NaCl were each optimized in concentration and volume, as confirmed by UV-Vis spectrophotometer and electron transmission microscope (TEM), NaCl and gli4 aptamer concentrations were optimized to 120 mM and 125 nM, respectively. When detecting gliadin based on optimization, the linear curve range (0-120 mg/L) that meets the gluten-free standard (20 mg/L) within 70 min, and the detection

limit is 10.5 mg/L (UV-Vis spectrophotometer) and 12 mg/L (colorimetric). In addition, as a selectivity test, the aptasensor was observed for other grain proteins (barley, oats, corn, and rice), and it was confirmed that it responded only to gliadin. To confirm the detection in real foods, ELISA and aptasensor were compared by spiking gliadin at any concentration into gluten-free foods (pasta, bread, and cookie), and it was shown that it could be detected below the gluten-free standard. Therefore, the results of this study are expected to be applied in the food industry to gluten-sensitive allergen patients and environments that require verification of gluten-free food.

Keywords: gluten, gliadin, gold nanoparticles, aptamers, label-free, colorimetric aptasensor, detection

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I. INTRODUCTION

Wheat, one of the three major crops used worldwide, along with corn and rice, contains gluten. Gluten, which consists of glutenin and gliadin, is an important raw material for determining the quality of dough foods such as bread and pasta by giving the dough viscoelasticity (Jessica R., 2016). However, gluten can cause celiac disease, a severe autoimmune disease (CD) in people who genetically cause damage to the small intestine. The prevalence of CD is reported to be 1% in North America and Europe (Katharina Anne Scherf, & Roland Ernest Poms, 2016). The only way to avoid CD is to avoid eating foods that contain gluten. However, there are not many practical methods for detecting gluten-free standards. The Codex defines the gluten-free standard as a gluten content of less than 20 ppm, and the deficient gluten standard is defined by the European Union (EU) as 20-100 ppm (Codex Standard 118-1979, 2008; Commission Implementing Regulation (EU) 828, 2014). Therefore, the fast and sensitive detection of gluten according to the criteria for gluten-containing grains proposed by Voluntary Incidental Trace Allergen Labeling (Vital 3.0) is critical in the food industry (Holzhauser et al., 2020).

Over the past decade, new methods of gluten detection have been

continuously developed along with various enzyme-linked immune sorbent assays (ELISA). Especially the detection methods such as real-time polymerase chain reaction (RT-PCR) (Jorge R. Mujico et al., 2011), gel-permeation high-performance liquid chromatography with fluorescence detection (GP-HPLC-FL) (Katharina Anne Scherf, Herber Wieser, & Peter Koehler, 2016), microarray (Maria Serena Chiriaco et al., 2015), liquid chromatography spectrometry (LC-MS) (Ahmed Gomaa, & Joyce Boye, 2015), and enzyme-linked immune sorbent assays (ELISA) (Carmen Diaz-Amigo, & Bert Popping, 2013; Alexandra Scharf et al., 2013; Gregory J. Tanner et al., 2013) have advantages of accuracy and sensitivity, but there are disadvantages that it is expensive, the detection time is long, and it is difficult to use. Therefore, developing a simple, fast, and inexpensive on-site portable detection method is still necessary.

Recently, detection methods using an aptamer as a biosensor have emerged for the detection of gluten. The aptamer is a small molecule with high affinity and specificity made from single-stranded nucleic acids through the SELEX (Darmostuk, Rimpelova, Gbelcova, & Ruml, 2015). Most of the gluten detection using aptamer is electrochemical and fluorescence analysis method (Amaya-González et.al., 2015; López-López et.al., 2017; Malvano et.al., 2017; Pla, Martínez-Bisbal et.al., 2021; Svigelj et.al., 2022; Svigelj et.al., 2022). However, these studies have many limitations in the need for

equipment, as the food field has to be easily detected on-site. Alternatively, colorimetric detection has many advantages as a field biosensor in that it can detect without complicated equipment. Colorimetric detection is used for food safety in the field because it has the advantages of simplicity and rapidity. Colorimetric detection using aptamers has been studied for chemicals such as aflatoxin B1, Hg(II), Fe(III), cadmium, kanamycin, T-2 toxin, zearalenone, H_2O_2, and glucose, but protein detection in food has not yet been studied (Guo, Zhang, Shao, Wang, Wang, & Jiang, 2014; Hu, Huang, Liu, & Liu, 2021; Li et al., 2021; Luan et al., 2015; Tripathy, Woo, & Han, 2013; Xing et al., 2022; Zhang et al., 2021; Zheng, Wu, Wang, Xiao, & Yu, 2022).

In this study, we have developed an easy-to-use, visible signal and inexpensive label-free aptasensor that enables on-site detection of gliadin in the food industry. This detection method included salt-induced aggregation of gold nanoparticles (AuNP) and aptamer, a nucleotide sequence that explicitly recognizes gliadin in the gluten of wheat-based protein. In addition, the developed aptasensor performed quantitative detection evaluation in wheat-based food samples, including pasta and bread, for field application.

II. MATERIALS AND METHODS

2.1. Materials

Chloroauric acid and HEPEs and Gliadin from wheat were purchased from Sigma Aldrich (St. Louis, MO, USA). Trisodium citrate was purchased from Yakuri Pure Chemicals Co., Ltd (Kyoto, Japan). The gli4 aptamer, 5'-CCAGTCTCCCGTTTACCGCGCCTACACATGTCTGAATGCC-3', was synthesized from Bioneer, Ltd (Seoul, Korea) (Amaya-Gonzalez, de-Los-Santos-Álvarez, Miranda-Ordieres, & Lobo-Castanon, 2014). Gliadin ELISA (R7001) was purchased from R-Biopharm (Darmstadt, Germany). The method used by the ELISA to detect gliadin is included in the supplementary information. The pasta was purchased from Dececco (Fara san martino, Italy). The bread was purchased from a Paris baguette (Seoul, Korea). Gluten-free pasta, bread, and cookie were purchased from Glfree (Hwaseong, Korea).

2.2. Instrumentation

Absorbance measurements were performed on a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan). The pH values of all buffer solutions were determined using the Professional Meter PP-15 (Satorious, Göttingen, Germany). Particle sizes and distributions were determined by

dynamic light scattering (DLS) with a Zetasizer Nano-ZS 90 (Malvern, Worcestershire, UK). Transmission electron microscopic (TEM) images were obtained using a LIBRA 120 transmission electron microscope (Karl Zeiss, Oberkochen, Germany). Grinding the food samples was performed with a Blendforce Glass-BL310EKR (Tefal, France).

2.3. Synthesis of gold nanoparticles (AuNP)

Gold nanoparticles (AuNP) were prepared by reduction with trisodium citrate, which is a commonly used method. Firstly, 5 mL of 10 mM H4AuCl4 solution and 45 mL of distilled water were boiled at 350 ℃ for 15 min. Then, 5 mL of 38.8 mM trisodium citrate was boiled at 250 ℃ for 30 min, and then cooled at room temperature. The particle size of AuNP was measured in the absorbance range of 515-550 using an ultraviolet/visible (UV/Vis) spectrophotometer and was determined using DLS (Dynamic light scattering).

2.4. Optimization of detection condition

2.4.1. Measurement of the absorbance

200 μL of AuNP (absorbance of 2.0 at 520.0 ± 0.5 nm, diameter of 13 nm) was incubated with 230 μL of HEPEs buffer (10 mM, pH 7.4). After incubation, the mixture was reacted with a 10 μL gli4 aptamer (6.25 μM) for

30 min. The final concentration of gli4 aptamer was 125 nm due to a 50-fold dilution. After incubation, 10 μL of various concentrations of gliadin (target) were added to the mixture and reacted for 30 min. After the reaction, 10 μL of NaCl was added to the mixture and reacted for 10 min, and the total volume was 500 μL. The measurement volume of the cuvette was 400 μL, and the absorbance was measured in the wavelength range of 350-800 nm (Chang, Chen, Zhao, Wu, Wei, & Lin, 2014). The absorbance values were measured for the aggregation and dispersion of AuNP at 520 nm and 620 nm. The absorbance ratio $A = A_{620}/A_{520}$ was expressed as the degree of aggregation of AuNP.

2.4.2. Optimization of NaCl concentrations

To optimize the NaCl concentration, NaCl concentrations were set in the ranges of 10, 20, 40, 60, 80, 100, 120, 140, 160, and 180 nM, respectively. After mixing 200 μL of gold nanoparticles and 230 μL of HEPES buffer (10 mM, pH 7.4), 10 μL of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 0.01 mM EDTA) was added and reacted for 30 min. Then, after adding 60% ethanol and incubating for 30 min, 50 μL of NaCl was added for each concentration and incubated for 10 min. The absorbance reflected the degree of aggregation of AuNP in the A_{620}/A_{520} ratio, and the degree of aggregation of AuNP was

observed using TEM images.

2.4.3. Optimization of gli4 aptamer concentrations

To optimize the gli4 aptamer concentration, gli4 aptamer concentrations were set in the ranges of 6.25, 12.5, 25, 50, 125, 250, and 500 nM, respectively. After mixing 200 μL of AuNP and 230 μL of HEPES buffer (10 mM, pH 7.4), 10 μL of gli4 aptamer of various concentrations was added and reacted for 30 min. Then, after adding 60% ethanol and incubating for 30 minutes, 50 μL of 1.2 μM NaCl was added and incubated for 10 min. The absorbance reflected the degree of aggregation of AuNP in the A_{620}/A_{520} ratio, and the appearance of AuNP and gli4 aptamer was observed using TEM images.

2.5. Detection of gliadin

The gliadin reagent was used to obtain a standard for the detection range of gliadin. 1 g of gliadin reagent was mixed with 10 mL 60% ethanol (1:10 v/v ratio, 30 min) in a water bath at 50℃, and gliadin was purified by centrifugation (revolution per minute, 8,000, 30 min) at room temperature. The extracted gliadin was diluted with 60% ethanol for each of various concentrations (2,500-fold, 3,200-fold, 4,000-fold, 5,000-fold, 6,500-fold,

8,000-fold, 10,000-fold, 16,000-fold, 32,000-fold, 64,000-fold, and 100,000-fold). After mixing 200 μL of AuNP and 230 μL of HEPEs buffer (10 mM, pH 7.4), 10 μL of gli4 aptamer (6.25 μM) was added and reacted for 30 min. Then, after adding 10 μL of various concentrations of gliadin and incubating for 30 min, 50 μL of 1.2 μM NaCl was added and incubated for 10 min. The absorbance reflected the degree of aggregation of AuNP in the A_{620}/A_{520} ratio. For the quantification of gliadin and validation of the developed aptasensor, it was confirmed by the certified method, the ELISA (R-Biopharm) for the detection of gliadin.

2.6. Selectivity of the colorimetric aptasensor of gliadin

Selectivity is one of the important indicators in a detection system. In the detection system, selectivity test was conducted using corn, barley, oats, and rice, which are grains similar to wheat protein. In the same experimental conditions as presented above, the target conditions (corn, barley, oats, and rice) were set to a concentration of 300-fold dilution and a volume of 10 μL instead of gliadin.

2.7. Validation of gliadin detection in real foods

To confirm the performance of the aptasensor in real food, it was extracted from gluten-free foods. First, 1 g of gluten-free samples (pasta, bread, and cookie) were finely ground with a grinder and extracted with 10 mL of 60% ethanol (1:10 ratio v/v, 30 min) in a water bath at 50°C. The extracted samples were purified by centrifugation (revolution per minute, 8,000, 30 min) at room temperature. The extracted gluten-free samples (pasta, bread, and cookie) were diluted with 60% ethanol. The extracted samples were diluted 20-fold with 60% ethanol.

We validated spiked gluten-free samples (pasta, bread, and cookie) by comparing the developed aptasensor with a certified ELISA (R-Biopharm). The detection process for gluten-free samples (pasta, bread, and cookie) spiked with gliadin were the same as the method previously optimized for gliadin detection. After mixing 200 μL of AuNP and 230 μL of HEPEs buffer (10 mM, pH 7.4), 10 μL of gli4 aptamer (6.25 μM) was added and reacted for 30 min. Then, after adding 10 μL of gliadin (15, 30, and 60 ng/mL) and incubating for 30 min, 50 μL of 1.2 μM NaCl was added and incubated for 10 min. The absorbance reflected the degree of aggregation of AuNP in the A_{620}/A_{520} ratio. The gluten-free samples (pasta, bread, and cookie) spiked with gliadin were quantitatively compared with the developed aptasensor and a

certified method, ELISA.

2.8. Statistical analysis

The data represent an average of at least three independent experiments or measurements, and the results are expressed as mean ± standard deviation (SD).

III. RESULTS AND DISCUSSION

3.1. Overall detection procedures

In this study, an on-site colorimetric detection was proposed to easily and quickly detect gliadin, a representative protein allergen in food. This on-site detection involves a simple gliadin extraction process and the aptasensor, an AuNP-based colorimetric detection method (Fig. 1). First, homogenize 1 g of food samples such as bread, noodles, and cookie and extract (50℃ for 30 min) in 10 mL of 60% ethanol. After that, impurities are removed by centrifugation (x 8,000 g and 30 min) to obtain gliadin, water-soluble protein, extracted in the supernatant. This extraction method was carried out in ethanol solvent and slightly elevated temperature (50℃) for easy field use. In addition, to satisfy the gluten-free standard, we selected gliadin, which constitutes gluten together with glutenin, as a biomarker since gliadin is soluble in ethanol, but glutenin is insoluble. Therefore, it was attempted to satisfy the gluten-free standard by doubling the detected gliadin content (Scherf & Poms, 2016). Gliadin, which was extracted from food samples could be quantitatively detected with the aptasensor, an AuNP-based colorimetric detection method. The principle of this detection method exploits the correlation among AuNP, the target gliadin, and the gli4 aptamer. The citrate-capped AuNP could ensure

colloidal stability (well dispersed), caused by electrostatic attraction due to opposing charges (Epanchintseva, Vorobjev, Pyshnyi, & Pyshnaya, 2018). Furthermore, the addition of NaCl could control the colloidal stability of AuNP. In addition, since some of the bases of the Gli4 aptamer have a positive charge, the aptamer can be well adsorbed on the surface of the negatively charged AuNP, resulting in secured salt stability of AuNP. Therefore, in this aptasensor, AuNP stabilized with gli4 aptamer compete with gliadin for the Gli4 aptamer, which could quantitatively identify the gliadin according to the AuNP aggregation (A_{620}/A_{520}), caused by NaCl, using absorbance or through color change (red to purple) with the naked eye (Gopinath, Lakshmipriya, & Awazu, 2014).

An essential point in the aptasensor for gliadin detection was to determine the reaction sequence of the gliadin and gli4 aptamer to AuNP. Therefore, the reaction sequence of the gliadin and gli4 aptamer to AuNP was optimized. As shown in Fig. 2, When gliadin and gli4 aptamer were sequentially added to AuNP, no change in absorbance was observed due to aggregation of AuNP. Unlike the target (heavy metals, toxins) of previous studies, this non-aggregation phenomenon was expected to be blocked on the AuNP surface due to the large size of gliadin as a protein. To overcome these difficulties, we changed the reaction sequence to make the AuNP surface less

susceptible to gliadin influence. Therefore, the assay was finally decided on binding the gli4 aptamer to the AuNP and then adding the target.

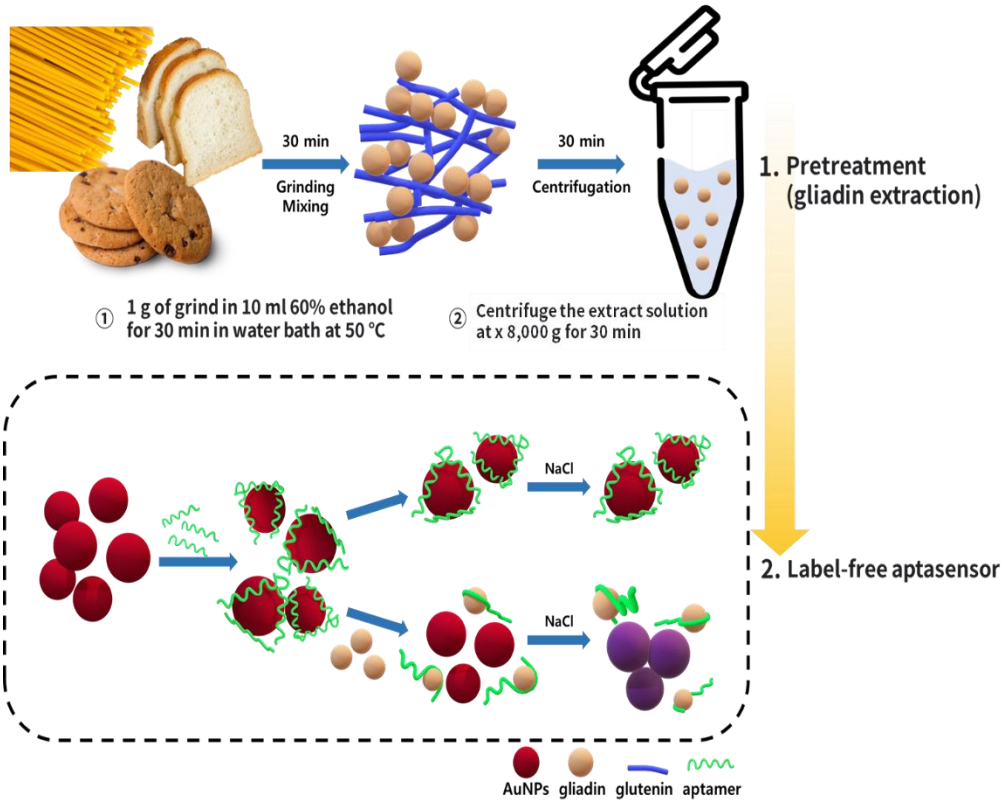

Figure 1. Schematic diagram of aptamer-based colorimetric detection using AuNP to detect gliadin. Gliadin extraction process from food and aggregation of AuNP with and without gliadin.

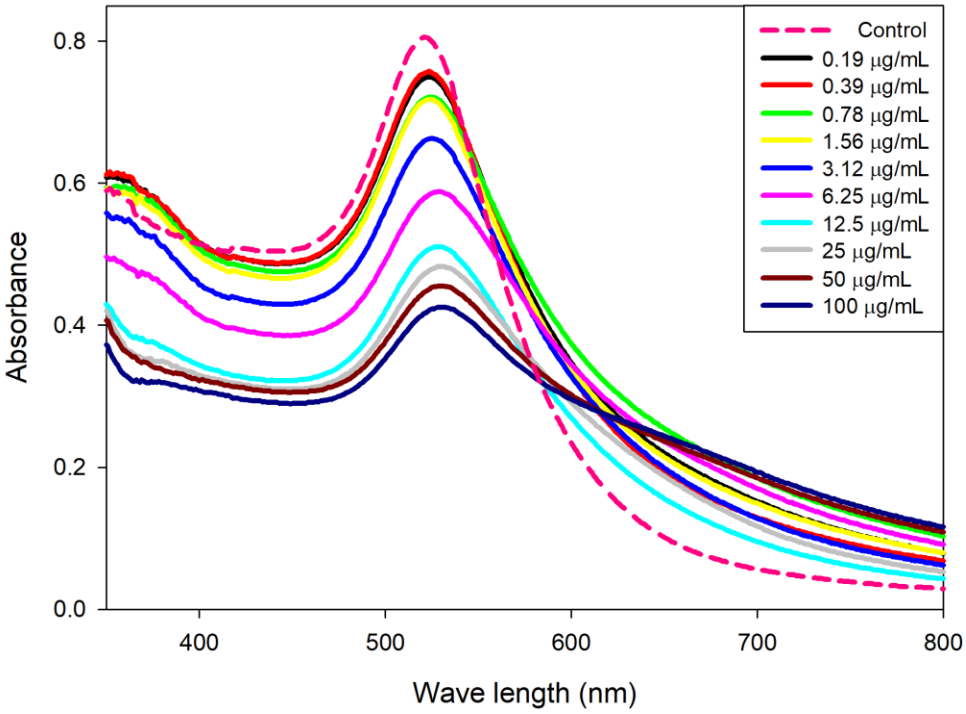

Figure 2. The absorbance according to the binding order of aptamers after gliadin binding to AuNP.

3.2. Optimization of detection condition

3.2.1. Optimization of NaCl concentrations

The citrate-capped AuNP had a repulsive force with a negative charge but was neutralized when NaCl was added. However, due to the sodium ions present in NaCl, AuNP was not well dispersed, and aggregation occurred. The degree of aggregation depended on the different NaCl concentrations (Fig. 3). When the NaCl concentration was low, the AuNP was dispersed without aggregation due to the aptamer on the surface. On the other hand, when the NaCl concentration was high, it exceeded the ability of the aptamer to protect the AuNP surface, so an appropriate concentration was required.

The degree of aggregation was observed with A_{620}/A_{520} with NaCl concentrations ranging from 20 to 160 mM at 20 mM intervals (Fig. 4). There was a change at low concentration (20 – 80 mM), but it was not significant and showed a considerable change from 100 mM. The concentration did not affect the AuNP surface charge to be neutralized. The NaCl concentration was observed to be a saturated concentration from 140 mM, and the 120 mM concentration was the concentration before saturation and showed an enormous change (Fig. 5). The aptamer could not protect the AuNP surface when used above the NaCl saturation concentration. In addition, when naked

AuNPs and added 120 mM NaCl were compared, absorbance and TEM images confirmed the aggregation of AuNPs with 120 mM NaCl (Fig. 7(b) and 8(b)). Therefore, an appropriate concentration of NaCl was required, and a concentration of 120 mM was optimally selected.

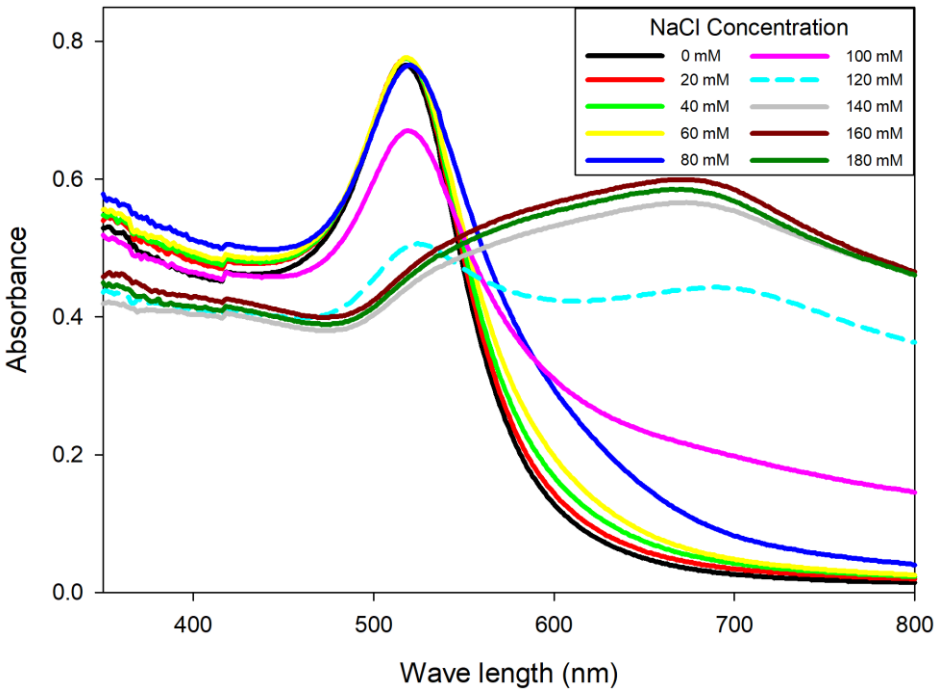

Figure 3. The absorbance of AuNP according to various NaCl concentrations.

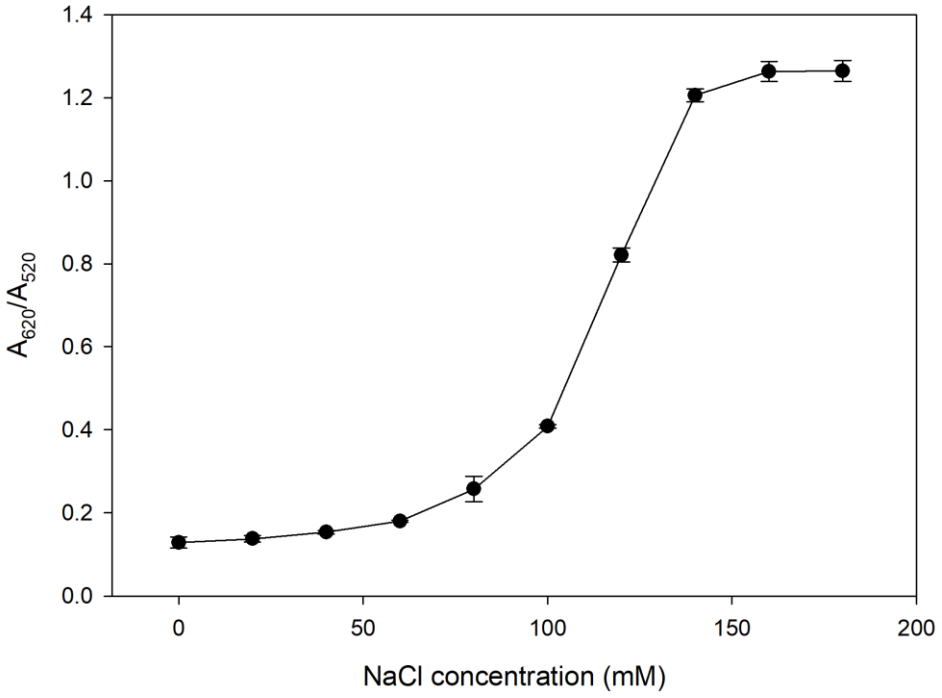

Figure 4. The aggregation degree ratio (A_{620}/A_{520}) of AuNP according to various NaCl concentrations.

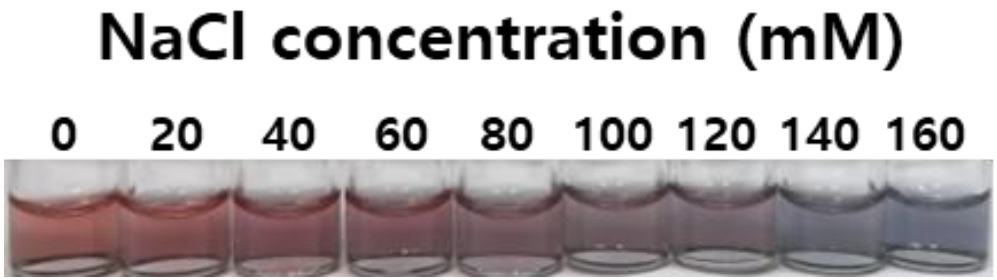

Figure 5. The color change of AuNP according to various NaCl concentrations.

3.2.2. Optimization of gli4 aptamer concentrations

To maximize the sensitivity of this assay, it was necessary to determine the optimal aptamer concentration along with NaCl optimization. When the glia4 aptamer is relatively less, the AuNP surface was not protected resulting in salt inducing aggregation; conversely, when there were too much aptamers, the binding between the aptamer on the AuNP surface and the target was difficult due to competition between the aptamer adsorbed on the AuNP surface and the dispersed aptamer. Therefore, the optimal aptamer concentration was required. The aggregation pattern according to AuNP and the aptamer concentration, which increased two-fold from 6.25 nM to 500 nM, was shown in Fig. 6 (adding 120 mM of NaCl). In the aptamer concentration range of 6.25 to 50 nM, less aggregation occurred than in the absence of aptamer. On the other hand, the aggregation of AuNP did not occur in the aptamer concentration range of 125 to 500 nM. Therefore, we identified aggregation patterns at selected concentrations of AuNP, gli4 aptamer, and NaCl to determine the optimal aptamer concentration. As shown in Fig. 7, the aggregation degree of four samples was analyzed by spectrophotometer: naked AuNP, AuNP and 12 mM NaCl, AuNP and 125 nM aptamer, and AuNP, 125 nM aptamer, and 120 mM NaCl. It was confirmed that gli4 aptamer at a concentration of 125 nM could protect AuNPs well under the condition of 120

mM NaCl; this concentratio of NaCl could aggregate the naked AuNP. In addition, as a result of the aggregation pattern through TEM analysis, it was confirmed that AuNPs adsorbed with 125 nM aptamer showed a little aggregation, but the AuNP could secure dispersion stability (Fig. 8). Therefore, a concentration of 125 nM was selected as the optimal aptamer concentration.

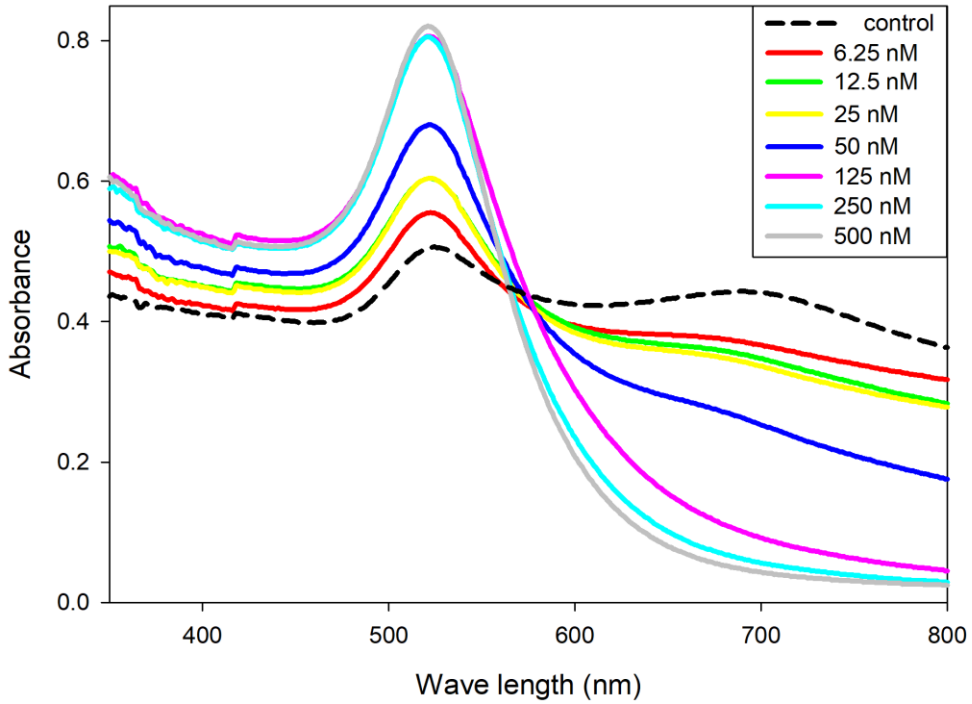

Figure 6. The absorbance of AuNP with various gli4 aptamer concentrations.

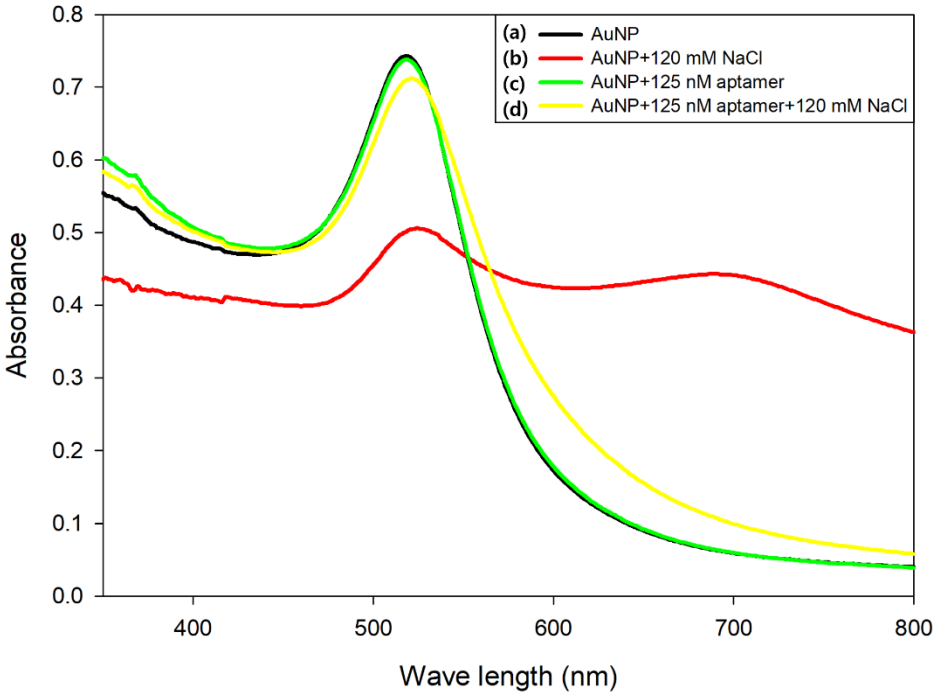

Figure 7. The absorbance under 4 different samples based on AuNP : (a) AuNPs; (b) AuNPs + 120 mM NaCl; (c) AuNPs + 125 nM Aptamer; (d) AuNP + 125 nM Aptamer + 120 mM NaCl.

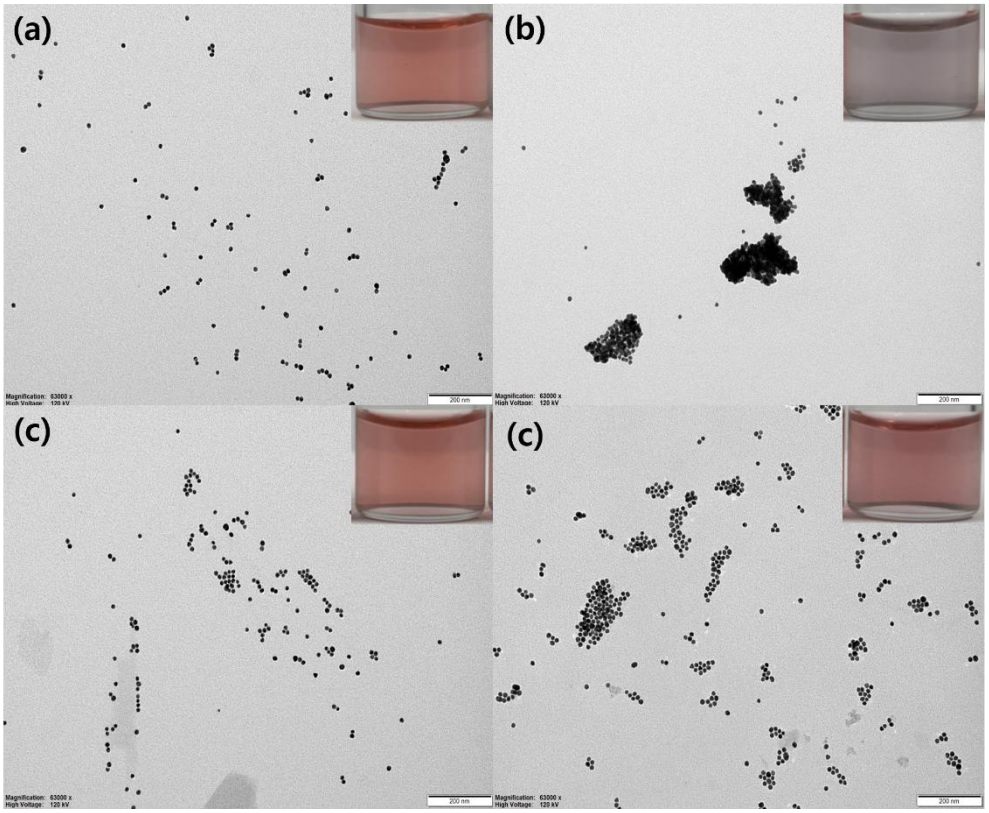

Figure 8. The TEM images of 4 different samples based on AuNP : (a) AuNP; (b) AuNP + 120 mM NaCl; (c) AuNP + 125 nM Aptamer; (c) AuNP + 125 nM Aptamer + 120 mM NaCl.

3.3. Detection of gliadin

We evaluated the detection limit and concentration range of the developed label-free colorimetric detection method with the standard gliadin reagent. The gliadin standard reagent was prepared as a sample by extraction and dilution, and the AuNP aggregation pattern was analyzed through spectrophotometer with an optimized aptamer and NaCl at a gliadin concentration from 0 to 300 ng/mL (Fig. 9). We plotted the absorbance ratio (A_{620}/A_{520}) to quantify the change in absorbance, and the linear equation was $y = 0.0028x + 0.2385$ in the linear curve (from 0 to 300 ng/mL of gliadin concentration), and the correlation coefficient was 0.9916 (Fig. 10). The gliadin reagent was extracted 1:10 (10-fold dilution) and the extracted gliadin was diluted 20-fold, a total of diluted 200-fold. The original gluten-free standard was 10 mg/L, but the gliadin was diluted 200-fold and converted to a gluten-free standard suitable for concentration in the aptasensor. The gliadin concentration range expressed by the linear equation included the concentration of a gluten-free standard (50 ng/mL), and the detection limit of this assay was appeared as a level of 26.3 ng/mL (10.5 mg/L at 20 mg/L on gluten-free standard). In addition, as shown in Fig. 11, we confirmed the detection limit and range that could be distinguished with the naked eye; the color change was recognized in the 30 ng/mL gliadin sample, which showed

a value below the gluten-free standard. The gliadin sample at 96 ng/mL near 100 ng/mL, which is a low gluten-free standard, was able to clearly recognize a color change

We compared the developed colorimetric aptasensor method with other previous methods of gliadin detection, and our method has advantages in terms of detection limit and equipment. This colorimetric aptasensor detection was simple, cost-effective, and applicable to on-site detection compared to the article described in a variety of methods (Table. 1). In addition, the advantage of the developed aptasensor is significant in detecting gliadin in gluten with the naked eye without using any equipment.

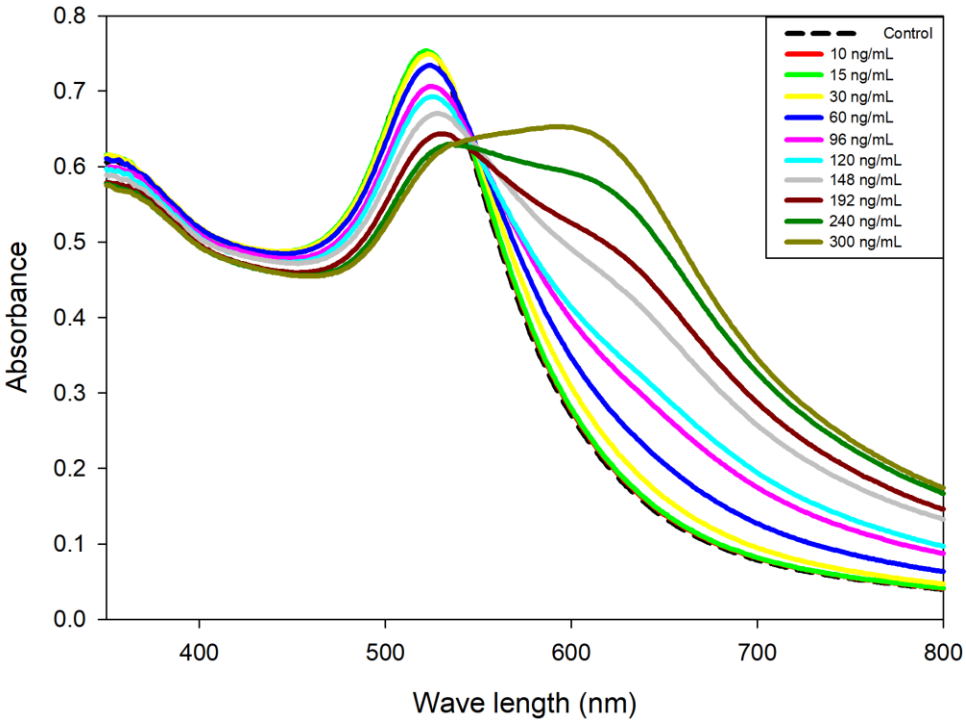

Figure 9. The absorbance of AuNP at various gliadin concentrations.

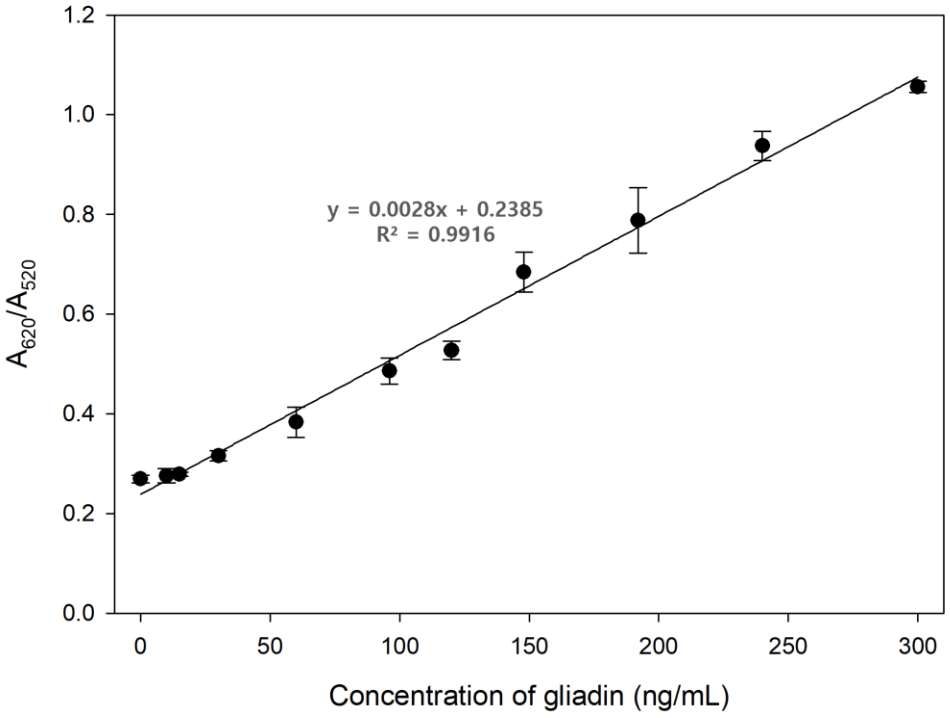

Figure 10. The aggregation ratio (A_{620}/A_{520}) of AuNP according to various gliadin concentrations.

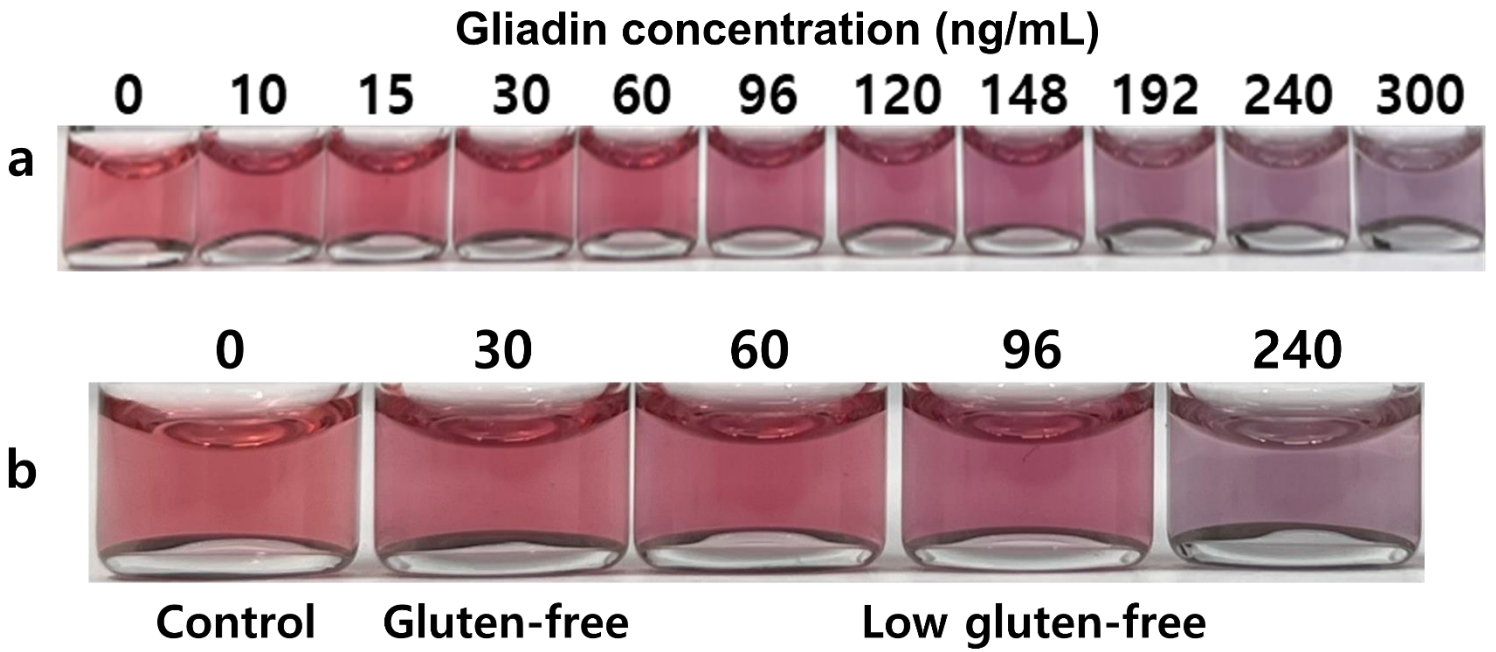

Figure 11. The AuNP color change according to gliadin concentration. (a) The visual color change at various gliadin concentrations. (b) The color change for detection at gluten-free and low gluten-free concentrations of gliadin.

Table 1. Comparison of the developed method in this work with other published detection methods for the detection of gliadin

Method	Limit of detection	Instrument	Sample type	Refs.
Real-time apta-PCR	100 µg/L	PCR	Gliadin solution	Pinto et al., 2014
Paper-based aptamer-antibody sandwich	0.2 mg/L	Impedance spectroscopy	Gliadin solution, Gluten-free flour, corn flakes	Svigelj, Dossi, Grazioli, & Toniolo, 2022
Impedimetric sensing of immune-enzymatic	5 mg/L	Electrochemical impedance spectroscopy (EIS)	Gliadin solution	Bottari, Moretto, & Ugo, 2018
Impedimetric aptasensor	5 mg/L	Electrochemical impedance spectroscopy (EIS)	Beer, Rice, Corn flour	Malvano, Albanese, Pilloton, & Di Matteo, 2017
Aptamer based colorimetric biosensor	10.5 mg/L 12 mg/L	UV-Vis spectrophotometer Not used	Gliadin solution Pasta, Bread, Cookie	This work

3.4. Selectivity of the colorimetric aptasensor of gliadin

To determine the selectivity of the label-free colorimetric aptasensor with respect to gliadin, we used the proposed method to test control samples (only PBS) and grain foods including oat, corn, rice, and barley. To evaluate field applicability by identifying the potential risk that the aptasensor system would be inhibited by potential factors in real food, we performed selectivity experiments using cereal foods containing various allergen proteins. As shown in Fig. 12, the absorbance ratio (A_{620}/A_{520}) representing the aggregation of AuNP showed a higher value (1.14) in the gliadin sample than in other grain foods. In addition, even in the actual color change, a significant color change was observed only in the gliadin sample. The α2-gliadin sequences is capable of binding toxic and non-toxic proteins other than wheat (gliadin), such as rye (secalins), barley (hordeins), rice, maize, and oats (avenins), and the gli4 aptamer is known to have the highest affinity with gliadin among the α2-gliadin sequences (Shan et al., 2002). In this selectivity evaluation, it was determined that the gliadin detection aptasensor including the gli4 aptamer was not affected by other grains including oats, corn, rice, and barley. Therefore, it was possible to secure the reliability in terms of selectivity for gliadin detection.

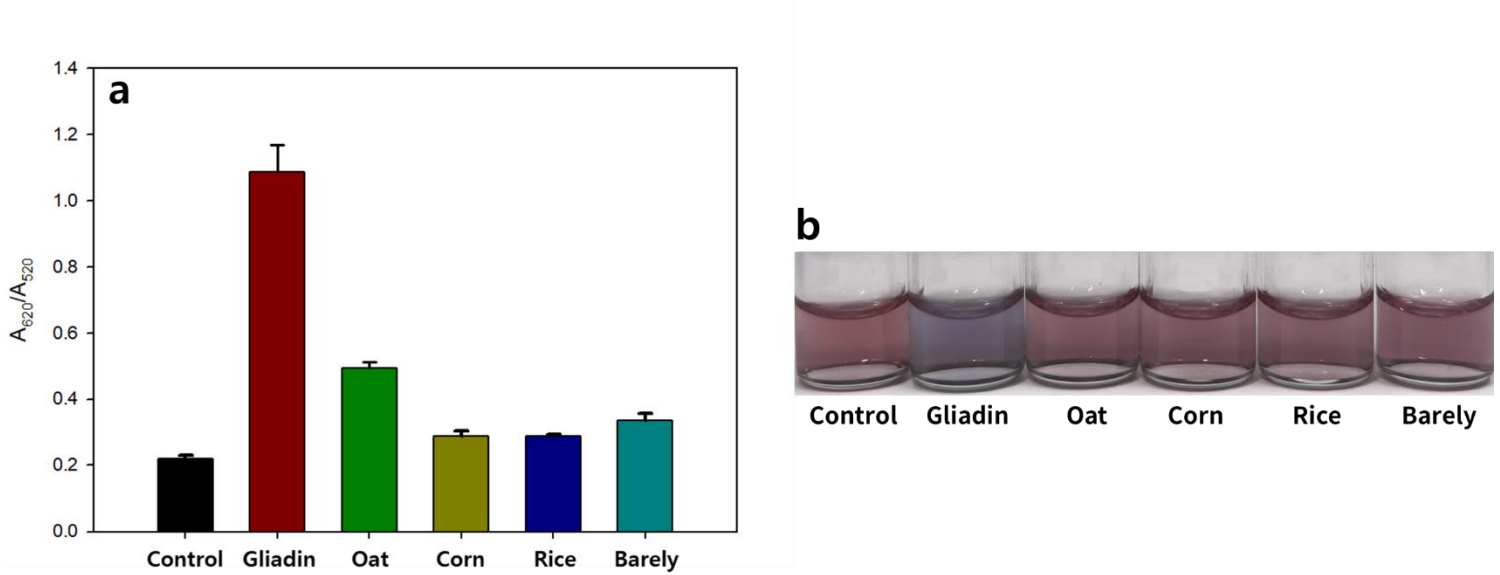

Figure 12. The selectivity with oat, corn, rice, and barely in the colorimetric aptasensor detection method for gliadin detection. (a) The aggregation degree ratio of AuNP (A_{620}/A_{520}) according to various samples. (b) The color change of AuNP according to various samples.

3.5. Validation of gliadin detection in real foods

To confirm the gluten detection of the aptasensor, real foods (pasta, bread, and cookie) marked gluten-free were selected as samples. The performance of the colorimetric aptasensor developed was compared with a certified ELISA (R-Biopharm) by spiking gliadin into a gluten-free sample. To verify accuracy and practical application, gluten-free food samples (pasta, bread, and cookie) were conducted with spiked gliadin (15, 30, and 60 ng/mL) (Table. 2). Both the aptasensor and ELISA, which was not detected in gluten-free samples (pasta, bread, and cookie). The overall recovery rates of aptasensor and ELISA were 90.1-103.9% and 83.1-95.2% in gluten-free samples (pasta, bread, and cookie) with spiked gliadin, respectively. These results were significant for the aptasensor to quantitatively distinguish gluten-free foods with spiked gliadin.

The recently announced Voluntary Incidental Trace Allergen Labeling (VITAL) is an effective way for food producers to minimize risks to consumers with allergies. It is a criterion to help determine whether an allergen is present from unavoidable cross-contamination. According to the VITAL® 3.0 reference dose, doses of 0.7 mg/kg and 6.1 mg/kg of wheat protein can induce allergic reactions in 1% and 5% of the wheat allergic population (ED01 and ED05) (Remington et al., 2020). At least less than 7 mg/kg of wheat may

be included, as wheat has a conversion factor of 10 to an allergenic food (Holzhauser et al., 2020). The detection limit of gliadin was 22 mg/kg, which satisfies safety criteria at 61 mg/kg of ED05. This result was meaningful in that it provides a basis for the on-site detection of gliadins in wheat proteins by an aptasensor detection system.Amaya-Gonzalez, S., de-Los-Santos-Álvarez, N., Miranda-Ordieres, A. J., & Lobo-Castanon, M. J. (2014). Aptamer binding to celiac disease-triggering hydrophobic proteins: a sensitive gluten detection approach. Analytical chemistry, 86(5), 2733-2739.

Table 2. Comparison of recovery test for gliadin spiked food samples

Spiked concentration (ng/mL)	Samples	ELISA (ng/mL)	Recovery (%)	Aptasensor (ng/mL)	Recovery (%)
0	Gluten-free pasta Gluten-free bread Gluten-free cookie	N.D	-	N.D	-
15.0	Spiked gluten-free pasta	15.8 ± 2.4	95.2	15.8 ± 3.3	95.0
	Spiked gluten-free bread	16.0 ± 1.0	93.7	15.8 ± 3.0	90.1
	Spiked gluten-free cookie	18.1 ± 1.6	83.1	16.6 ± 2.7	94.9
30.0	Spiked gluten-free pasta	31.5 ± 4.9	95.2	29.3 ± 1.9	102.3
	Spiked gluten-free bread	32.0 ± 1.9	93.7	28.9 ± 0.4	95.8
	Spiked gluten-free cookie	36.1 ± 3.2	83.1	31.3 ± 0.5	103.9
60.0	Spiked gluten-free pasta	63.0 ± 9.8	95.2	61.8 ± 0.4	97.0
	Spiked gluten-free bread	64.1 ± 3.8	93.7	62.0 ± 2.2	102.9
	Spiked gluten-free cookie	72.2 ± 6.5	83.1	58.3 ± 1.3	96.8

a Data are mean values of triplicate measurements. Values are shown as means ± standard deviation.

CONCLUSION

Celiac disease is a disease that causes fatal damage to the human body's digestion by the gliadin in the gluten protein in wheat foods. So far, the effective method for celiac disease was gluten-free diet, but establishments and restaurants that handle gluten-containing foods may be contaminated with gluten-free foods. It was a difficult task to easily and quickly detect gluten contamination in the field. Here, we developed a colorimetric aptasensor for on-site detection of gliadin in gluten using a gli4 aptamer based on AuNP aggregation. The naked-eye detection of gliadin has not been reported much, and the result is qualitative detection of gliadin by eye, and quantitative detection by UV-vis absorption spectroscopy. This assay detected gliadin with an LOD of 10.5 mg/L and a linear detection range of 0-120 mg/L within 70 min, which was also distinguishable by the naked eye at 12 mg/L. Additionally, as a detection method applicable to the food industry, it was achieved below the gluten-free standard in pasta and bread in real food. The advantages of this assay simplicity, practicality, selectivity, quickness, and cost-effectiveness are alternatives to existing gluten detection methods, but the signal needs to be further improved in this aptasensor system. In addition, further studies are needed to determine whether it can be applied to the detection of proteins and

microorganisms other than gliadin in food groups. The colorimetric aptasensor is a useful tool for food safety in the field, as a field sensor for gluten measurement in food for patients with celiac disease.

REFERENCES

Amaya-Gonzalez, S., de-Los-Santos-Álvarez, N., Miranda-Ordieres, A. J., & Lobo-Castanon, M. J. (2014). Aptamer binding to celiac disease-triggering hydrophobic proteins: a sensitive gluten detection approach. Analytical chemistry, 86(5), 2733-2739.

Biesiekierski, J. R. (2017). What is gluten? Journal of gastroenterology and hepatology, 32, 78-81.

Bottari, F., Moretto, L. M., & Ugo, P. (2018). Impedimetric sensing of the immuno-enzymatic reaction of gliadin with a collagen-modified electrode. Electrochemistry Communications, 97, 51-55.

Chang, C.-C., Chen, C.-Y., Zhao, X., Wu, T.-H., Wei, S.-C., & Lin, C.-W. (2014). Label-free colorimetric aptasensor for IgE using DNA pseudoknot probe. Analyst, 139(13), 3347-3351.

Chiriacò, M. S., de Feo, F., Primiceri, E., Monteduro, A. G., de Benedetto, G. E., Pennetta, A., . . . Maruccio, G. (2015). Portable gliadin-immunochip for contamination control on the food production chain. Talanta, 142, 57-63.

CODEX, S. (2008). STAN 118-1979. Standard for foods for special dietary use for persons intolerant to gluten.

Commission, E. (2014). Commission implementing regulation (EU) No 828/2014 of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. Off J Eur Union, 228, 5-8.

Darmostuk, M., Rimpelova, S., Gbelcova, H., & Ruml, T. (2015). Current approaches in SELEX: An update to aptamer selection technology. Biotechnology advances, 33(6), 1141-1161.

Diaz-Amigo, C., & Popping, B. (2013). Accuracy of ELISA detection methods for gluten and reference materials: a realistic assessment. Journal of agricultural and food chemistry, 61(24), 5681-5688.

Epanchintseva, A., Vorobjev, P., Pyshnyi, D., & Pyshnaya, I. (2018). Fast and strong adsorption of native oligonucleotides on citrate-coated gold nanoparticles. Langmuir, 34(1), 164-172.

Gomaa, A., & Boye, J. (2015). Simultaneous detection of multi-allergens in an incurred food matrix using ELISA, multiplex flow cytometry and liquid chromatography mass spectrometry (LC–MS). Food Chemistry, 175, 585-592.

Gopinath, S. C., Lakshmipriya, T., & Awazu, K. (2014). Colorimetric detection of controlled assembly and disassembly of aptamers on unmodified gold nanoparticles. Biosensors and Bioelectronics, 51,

115-123.

Guo, Y., Zhang, Y., Shao, H., Wang, Z., Wang, X., & Jiang, X. (2014). Label-free colorimetric detection of cadmium ions in rice samples using gold nanoparticles. Analytical chemistry, 86(17), 8530-8534.

Holzhauser, T., Johnson, P., Hindley, J. P., O'Connor, G., Chan, C.-H., Costa, J., . . . Miani, M. (2020). Are current analytical methods suitable to verify VITAL® 2.0/3.0 allergen reference doses for EU allergens in foods? Food and Chemical Toxicology, 145, 111709.

Khansili, N., Rattu, G., & Krishna, P. M. (2018). Label-free optical biosensors for food and biological sensor applications. Sensors and Actuators B: Chemical, 265, 35-49.

Loveday, S. M. (2020). Plant protein ingredients with food functionality potential. Nutrition Bulletin, 45(3), 321-327.

Malvano, F., Albanese, D., Pilloton, R., & Di Matteo, M. (2017). A new label-free impedimetric aptasensor for gluten detection. Food Control, 79, 200-206.

Mujico, J. R., Lombardía, M., Mena, M. C., Méndez, E., & Albar, J. P. (2011). A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. Food Chemistry, 128(3), 795-801.

Pinto, A., Polo, P. N., Henry, O., Redondo, M., Svobodova, M., & O'Sullivan, C. K. (2014). Label-free detection of gliadin food allergen mediated by real-time apta-PCR. Analytical and bioanalytical chemistry, 406(2), 515-524.

Ramalingam, S., Elsayed, A., & Singh, A. (2020). An electrochemical microfluidic biochip for the detection of gliadin using MoS2/graphene/gold nanocomposite. Microchimica Acta, 187(12), 1-11.

Scharf, A., Kasel, U., Wichmann, G., & Besler, M. (2013). Performance of ELISA and PCR methods for the determination of allergens in food: an evaluation of six years of proficiency testing for soy (Glycine max L.) and wheat gluten (Triticum aestivum L.). Journal of agricultural and food chemistry, 61(43), 10261-10272.

Scherf, K. A., & Poms, R. E. (2016). Recent developments in analytical methods for tracing gluten. Journal of Cereal Science, 67, 112-122.

Scherf, K. A., Wieser, H., & Koehler, P. (2016). Improved quantitation of gluten in wheat starch for celiac disease patients by gel-permeation high-performance liquid chromatography with fluorescence detection (GP-HPLC-FLD). Journal of agricultural and food chemistry, 64(40), 7622-7631.

Svigelj, R., Dossi, N., Grazioli, C., & Toniolo, R. (2022). based aptamer-antibody biosensor for gluten detection in a deep eutectic solvent (DES). Analytical and bioanalytical chemistry, 414(11), 3341-3348.

Tanner, G. J., Colgrave, M. L., Blundell, M. J., Goswami, H. P., & Howitt, C. A. (2013). Measuring hordein (gluten) in beer–a comparison of ELISA and mass spectrometry. PLoS One, 8(2), e56452.

Wu, Y.-Y., Huang, P., & Wu, F.-Y. (2020). A label-free colorimetric aptasensor based on controllable aggregation of AuNPs for the detection of multiplex antibiotics. Food Chemistry, 304, 125377.

Zhang, W., Wang, Y., Nan, M., Li, Y., Yun, J., Wang, Y., & Bi, Y. (2021). Novel colorimetric aptasensor based on unmodified gold nanoparticle and ssDNA for rapid and sensitive detection of T-2 toxin. Food Chemistry, 348, 129128.

국문초록

글루테닌과 글리아딘으로 구성된 글루텐은 반죽에 점탄성을 부여하여 빵, 파스타 등 밀 식품의 품질을 결정짓는 중요한 원료이다. 그러나 글루텐 단백질의 글리아딘은 소장에 치명적인 손상을 주는 셀리악 병 및 밀 알러젠을 유발되며, 이를 검출하기 위해 많은 방법들이 개발되었다. 하지만 연구된 검출 방법들은 복잡한 장비, 긴 시간, 비용으로 인해 현장에서 실용적으로 사용되기 어려움이 있다. 본 논문에서는 셀리악 병의 원인이 되는 글루텐에 존재하는 글리아딘을 현장에서 검출이 간편하고 장비가 필요 없이 육안으로 식별이 가능한 라벨 없는 압타센서 검출법을 개발하였다. 금 나노입자의 염 유도 응집을 기반으로 글리아딘을 특이적으로 인식하는 gli4 압타머를 활용하였다. 글리아딘의 전처리 과정은 추출하는 과정으로 에탄올 60%에 용해시켜 추출함으로써 현장에 적합하게 간소화했다. 금 나노입자의 염 응집 유도를 위해 gli4 압타머, 글리아딘 및 NaCl은 각각의 농도, 부피를 최적화하여, 분광광도계 및 전자 투과현미경(TEM)을 통해 NaCl 및 gli4 압타머 농도는 각각 120 mM, 125 nM로 선정했다. 최적화를 기반으로 글리아딘 검출시, 70분 이내로 글루텐 프리 기준(20mg/L)에서 선형 구간(0-

120 mg/L)과 검출 한계는 각각 10.5 mg/L (분광광도계), 12 mg/L(비색)로 검출되었다. 선택성 실험을 통하여 다른 곡물 단백질(귀리, 옥수수, 쌀, 보리)에 대해 관찰하였고 압타센서는 글리아딘에만 반응하는 것을 확인했다. 실제 식품에서의 검출을 확인하기 위해, 글루텐 프리 식품(파스타, 빵, 쿠키)에 글리아딘을 스파이킹하여 ELISA와 압타센서를 비교하였고 글루텐 프리 기준 이하로 정량화할 수 있음을 확인했다.

따라서 본 연구의 결과로 식품산업에서 글루텐에 민감한 알러젠 환자 및 글루텐 프리 식품의 검증이 필요한 환경에 적용이 될 수 있을 것으로 기대된다.

주요어: 글루텐, 글리아딘, 금 나노입자, 압타머, 라벨프리, 비색 압타센서, 검출법

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