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Article

The peroxidase-like activity of papain colorimetrically detects H_2O_2 and glucose with high sensitivity

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SUMMARY

To date, several glucometer methods are available for patient-guided glycemic control, which diminishes the onset and severity of diabetic complications. In a dipstick urine glucose test, horseradish peroxidase (HRP) oxidizes tetramethylbenzidine (TMB) in the presence of hydrogen peroxide (H_2O_2) to quantitatively produce a colorimetric signal and predict glucose concentration. Papain, a sulfhydryl protease secreted by the latex of *Carica papaya*, displays HRP-like activity with high selectivity. However, the current papain-based glucometer method lacks sensitivity. We hypothesized that catalyzing the oxidation of papain under varying buffer conditions (e.g., papain, TMB, and acetic acid concentrations) would improve glucose detection sensitivity. To test this, we performed glucose oxidase (GOx)-mediated glucose oxidation and papain-mediated TMB oxidation under varying sample conditions *in vitro* and spontaneously coupled them. The aims of this study were to validate the coupling of GOx-mediated glucose oxidation and papain-mediated TMB oxidation and identify the optimized conditions that maximize catalytic activity. We confirmed that glucose oxidase quantitatively produces H_2O_2 (in a glucose concentration-dependent manner), the presence of which catalyzes papain-mediated production of a colorimetric signal. When two oxidation reactions were coupled, we identified that the sensitivity of papain's glucose detection increased by ten-fold compared to previous studies with a markedly high correlation (R2 = 0.99). In particular, substituting sodium acetate-acetic acid (HAc-NaAc) buffer with acetic acid and increasing the sample incubation time and papain concentration markedly increased the sensitivity of colorimetric signals. The optimized protocols can be integrated into emerging hydrogel technology to develop a non-invasive glucometer patch predicting blood glucose from the patient's produced sweat.

INTRODUCTION

Diabetes mellitus (DM) is a clinically heterogeneous group of metabolic disorders characterized by prolonged, frequent episodes of hyperglycemia (1). Complex epigenetic interactions underlie DM with shared clinical findings of reduced insulin secretion and glucose utilization (2). Wide phenotypic variations occur by immunohistological profile, age, and ethnicity (3-5). Common etiologic distinctions include type 1 (immune-mediated beta cell destruction) and type 2 (relative insulin resistance and deficiency) DM (6). As of 2017, 229 million cases of DM were reported worldwide, and the worldwide prevalence of DM is dramatically rising owing to an increasingly sedentary lifestyle (7).

Prognosis-wise, glycemic control reduces the onset and severity of diabetic complications. Prolonged hyperglycemia is often related to nonenzymatic glycosylation, osmotic damage, and diabetic microangiopathy, increasing the incidence of multiorgan dysfunction (8). Hence, patient-guided measurement and management of preprandial and postprandial glucose are imperative. To date, the complete blood count (CBC) test, dipstick urine glucose test (DUGT), and finger/heel-prick blood glucose test (FBGT) are commercially available glucometric methods. CBC — with the necessity of a centrifuge separator, anticoagulant supplement, and basic cell count — is only accessible in private or academic practices (9). Most accessible are FBGTs, both commercially and by the ease of use (10, 11). Furthermore, there has been a novel application of continuous glucose monitoring (CGM) — insertion of sensing electrodes into the dermis via microneedle for the measurement of interstitial glucose levels (12).

Amongst glucometric methods, only DUGT provides colorimetric detection and is commercially available with low cost and high feasibility, though only accessible in hospital settings due to exclusion from Medicare and Medicaid (10, 13). It utilizes a subset of spontaneous reactions: glucose + O_2 → D-glucono-delta-lactone + hydrogen peroxide (H_2O_2); hydrogen peroxide + chromogen → oxidized chromogen + water (11). Glucose oxidase (GOx) catalyzes the oxidation of glucose, and quantitatively produced H2O2 yields hydroxyl radical (OH) via catalysis of peroxidase in a glucose concentration-dependent manner. Subsequently, OH oxidizes 3,3'5,5'-tetramethylbenzidine (TMB), which has a maximal absorption peak at 652 nm (**Figure 1**) (14).

Indirect colorimetric analysis of quantitatively produced H_2O_2 via horseradish peroxidase (HRP)-mediated TMB oxidation allows rapid, cost-effective, and feasible visual detection of glucose (15). HRP is most pervasively used

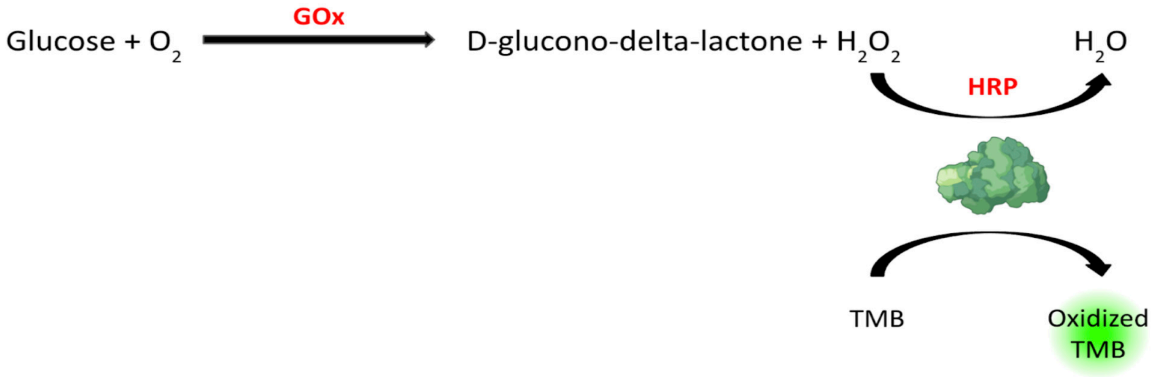

Figure 1. Schematic representation of standard colorimetric glucose detection. Glucose oxidase (GOx) oxidizes glucose to quantitatively produce H_2O_2, stimulating horseradish peroxidase (HRP)-mediated oxidation of tetramethylbenzidine (TMB) and the production of the colorimetric signal.

for H_2O_2 catalysis. In particular, HRP presents remarkable substrate specificity and catalysis efficacy, yielding accurate and selective results (16). However, as a naturally occurring catalyst, HRP is associated with several limitations, including sophisticated preparation, high cost, and low conformational stability in extreme conditions (17). Hence, there have been efforts to substitute HRP in enzymatic assays with nanomaterials or other natural enzymes, and the use of nanomaterials (noble metals and transition metal oxides) in the detection of glucose, H_2O_2, and uric acid has been reported (18). Despite promising results, nanomaterials suffer from poor biocompatibility and painstaking preparation expenditure (18).

Papain is a sulfhydryl protease secreted by the latex of *Carica papaya* (Spanish papaya). Its active site exists as a V-shaped cleft interfacing L- and R-domains – formed by cysteine from asparagine and glutamine residue – and participates in the catalysis of peptide hydrolysis (19). Owing to its high biocompatibility and marked degradation effect, papain is widely used in the food, medicine, and cosmetics industries (20). Remarkably, papain displays selective peroxidase-like activity by catalyzing the oxidation of TMB in the presence of H_2O_2 (14). In 2019, Chen et al. coupled the oxidation reactions of DUGT with the substitution of papain and proposed a novel colorimetric analysis for human urine samples (14). A significant correlation with high sensitivity was observed between varying concentrations of H_2O_2 and the absorbance at 652 nm (0–100 μM, R^2 = 0.994) (14). Nonetheless, the currently available papain-based glucometer method has low sensitivity in its reliable glucose detection range (0.1–0.5 mM), which impedes the method's incorporation into emerging glucometer platforms. "Sensitivity" described herein refers to the reliable detection range of H2O2 and glucose.

GOx catalyzes the oxidation of glucose, quantitatively producing H_2O_2 and D-glucono-delta-lactone (11). Under the presence of H2O2, papain catalyzes the oxidation of TMB, and oxidized chromogen produces a colorimetric signal with an absorption peak at 652 nm. Previous studies have confirmed the compatibility between GOx-mediated glucose oxidation and papain-mediated TMB oxidation in vitro. Thus, the coupled reactions can quantitively produce colorimetric signals under the presence of glucose, enabling sensitive detection of glucose concentration (14).

The literature currently suggests 100 μg/mL (0.0043 mM) papain, 5 mM TMB, and NaAc-HAc buffer for the catalysis of papain-mediated TMB oxidation (14). However, the current reliable glucose detection range (0.1–0.5 mM) lacks the sensitivity to be incorporated into any glucometer platform available. This experiment aimed to determine the optimized conditions that induce both oxidation reactions and to enable reliable glucose detection over a range that is useful for existing glucometer platforms.

Herein, we attempt to identify an optimized condition of GOx-mediated glucose oxidation and papain-mediated TMB oxidation to improve the papain-based glucometer method's reliable glucose detection range. We hypothesized that with modifications to reactions conditions, papain-mediated TMB oxidation would quantitatively produce colorimetric signals under varying H_2O_2 concentrations with improved sensitivity via quantitative oxidation of chromogen TMB, thus serving as a plausible, competent substitute for conventional HRP. We further hypothesized that the modified TMB oxidation reaction could be coupled with GOx-mediated glucose oxidation and optimized under varying buffer conditions to produce a sensitive colorimetric signal predicting sample glucose concentration. The GOx-mediated oxidation reaction was performed with varying concentrations of GOx, glucose, and phosphate-buffered saline (PBS). The papain-mediated oxidation reaction was replicated with varying concentrations of papain, TMB, H_2O_2, and acetic acid. Furthermore, the oxidation reactions were jointly catalyzed

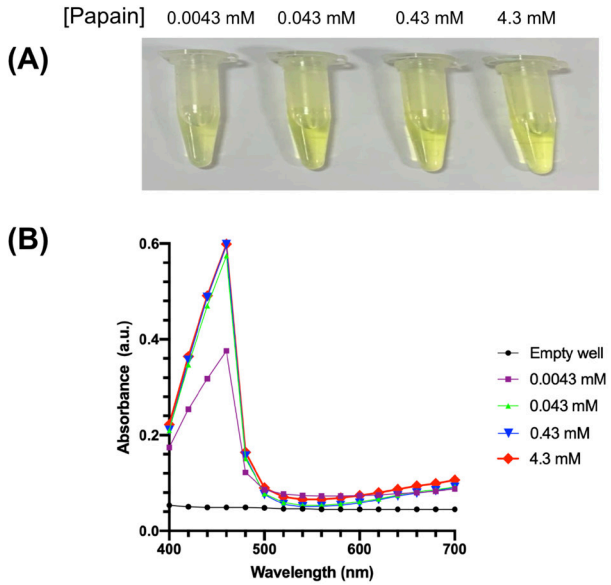

Figure 2. Papain catalyzes TMB in the presence of H_2O_2 and acetic acid buffer. (A) Samples under varying papain concentrations. **(B)** Absorbance at different wavelengths (400–-700 nm) under varying papain concentrations. Each sample containing 100 µL 5 mM TMB, 100 µL 88.2 mM H_2O_2, and 790 µL acetic acid under varying papain concentrations (0.0043–-4.3 mM, 10 µL/sample) was incubated at 40°C for 20 minutes. Maximal absorbance was observed at 460nm.

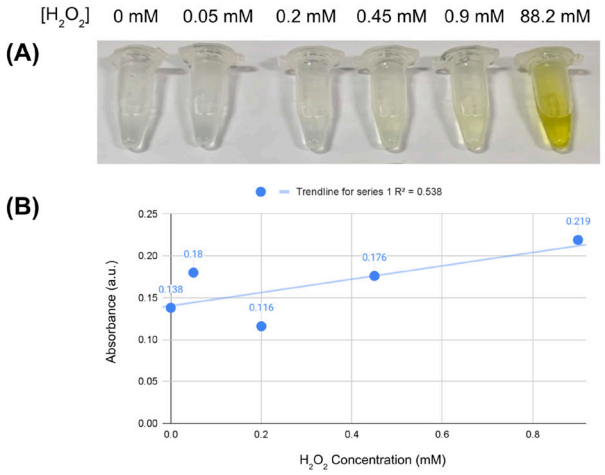

Figure 3. Papain-induced TMB oxidation in the presence of H_2O_2 increases absorbance at 460 nm. (A) Image of samples under varying H_2O_2 concentrations. **(B)** Linear regression shows a positive correlation between H_2O_2 concentration and absorbance at 460 nm (n=1). Each sample containing 10 µL 4.3 mM papain, 100 µL 5 mM TMB, and 790 µL acetic acid buffer under varying H_2O_2 concentrations (ranging from 0–88.2 mM, 100 µL/sample) was incubated at 40°C for 20 minutes. Coefficient of determination (R^2 = 0.538).

to analyze the viability and sensitivity of the total reaction mechanism. We report that coupled GOx-papain reactions yield colorimetric detection of glucose and H_2O_2 with stark sensitivity and accuracy; modifications to reaction buffer, papain, and reaction time were necessary for improvement in the reliable glucose detection range. The sensitive and colorimetric nature of the modified papain-based glucometer method highlights the versatility of which this method can be incorporated into emerging biomaterials for the development of a novel glucometer device.

RESULTS

We first evaluated the viability of papain-mediated TMB oxidation under constant H_2O_2 concentration. We utilized optimized conditions suggested by previous studies, but scaled down to the maximum volume capacity of a 1.5 mL Eppendorf tube (scaled by a 10:1 ratio, compared to the made volumes used in the previous study). This was done to reflect this study's further applications to hydrogel film, as the size (1 cm x 1 cm) of the film limits the volume of the reaction medium to 1.5 mL (14).

Each sample contained papain of varying concentrations (0.0043–4.3 mM), 5 mM TMB, 88.2 mM (3%) H_2O_2, and acetic acid buffer. All samples produced detectable colorimetric signals with an absorption peak of 460 nm, thus agreeing with the literature (4). We observed that the concentrations of papain and the absorbance at 460 nm in arbitrary units (a.u.) were positively correlated (**Figure 2**). In previous studies, the produced colorimetric signals were maximally absorbed at 652 nm, but in our work, maximum absorption occurred at 460 nm, partially due to HAc-NaAc buffer substitution by acetic acid (14). Hence, we performed a spectrophotometer at 460 nm in the following experiences, accounting for a shift in absorbance presumably owing to buffer substitution. The production of colorimetric signal accompanying papain-mediated TMB oxidation was viable and replicable in a scaled-down setting. From this experimental point, we consistently utilized 4.3 mM papain (1,000-fold from the literature-suggested concentration of 0.0043 mM), owing to the highly differentiable colorimetric signal produced under standard reaction conditions utilized herein (14). It was initially suggested and further validated that 4.3 mM papain would consistently catalyze the papain-mediated TMB oxidation under varying H_2O_2 concentrations with high sensitivity.

We then manipulated papain and TMB concentrations under varying concentrations of H_2O_2 to identify the optimized condition of papain-mediated TMB oxidation. Each sample contained 4.3 mM papain, 5 mM TMB, and acetic acid buffer under varying H_2O_2 concentrations ranging from 0 mM to 88.2 mM; the sample of 88.2 mM H_2O_2 functioned as a positive control. A trendline indicated a positive correlation between increasing H_2O_2 concentration and absorbance at 460 nm, but presented a weak coefficient of determination (R^2 = 0.538). In particular, samples of 0.05 mM and 0.2 mM

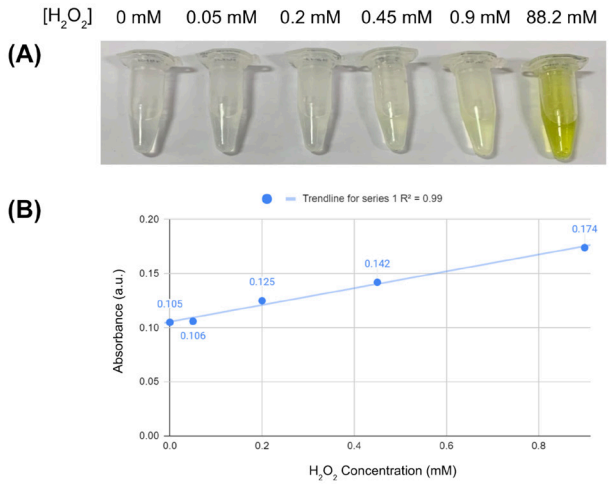

Figure 4. Increasing sample incubation time increases the correlation between H_2O_2 concentration and absorbance at 460 nm. (A) Image of samples under varying H_2O_2 concentrations. **(B)** Linear regression shows a positive correlation between H_2O_2 concentration and absorbance at 460 nm (n=1). Each sample containing 10 µL 4.3 mM papain, 100 µL 5 mM TMB, and 790 µL acetic acid buffer under varying H_2O_2 concentrations (ranging from 0–88.2 mM, 100 µL/sample) was incubated at 40°C for 30 minutes. Coefficient of determination (R^2 = 0.99).

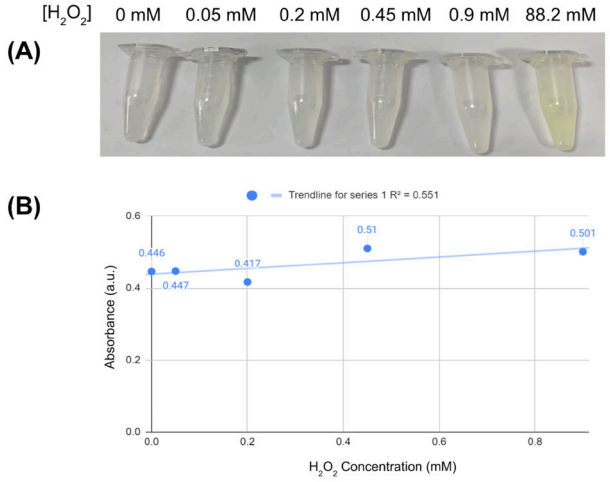

Figure 6. Increasing sample papain concentration ten-fold reduces correlation between H_2O_2 concentration and absorbance at 460 nm. (A) Image of samples under varying H_2O_2 concentrations. **(B)** Linear regression shows a positive correlation between H_2O_2 concentration and absorbance at 460 nm (n=1). Each sample containing 100 µL 4.3 mM papain, 100 µL 5 mM TMB, and 790 µL acetic acid buffer under varying H_2O_2 concentrations (ranging from 0–88.2 mM, 100 µL/sample) was incubated at 40°C for 30 minutes. Coefficient of determination (R^2 = 0.551).

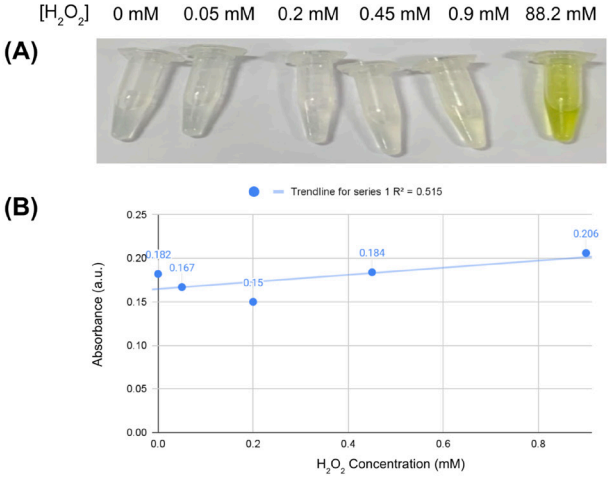

Figure 5. Increasing sample papain concentration two-fold reduces correlation between H_2O_2 concentration and absorbance at 460 nm. (A) Image of samples under varying H_2O_2 concentrations. **(B)** Linear regression shows a positive correlation between H_2O_2 concentration and absorbance at 460 nm (n=1). Each sample containing 20 µL 4.3 mM papain, 100 µL 5 mM TMB, and 790 µL acetic acid buffer under varying H_2O_2 concentrations (ranging from 0–88.2 mM, 100 µL/sample) was incubated at 40°C for 30 minutes. Coefficient of determination (R^2 = 0.515).

H_2O_2 displayed poor agreement with the trendline (**Figure 3**). This indicates that the TMB-produced colorimetric signal is a plausible predictor of H_2O_2 concentration but requires further optimization and modification from the literature-suggested conditions.

The previous trial was replicated with a prolonged incubation period from 20 to 30 minutes to enable sufficient catalysis of TMB by papain. The correlation between H_2O_2 concentration and absorbance at 460 nm was markedly significant and selective (R^2 = 0.99) (**Figure 4**). This confirms the compatibility of the optimized conditions previously described in the literature; hence, the substitution of NaAc-HAc buffer to acetic acid and a 1000-fold increase in papain concentration under prolonged incubation period allow a sensitive prediction within the detection range (14).

We further scaled the papain concentration two-fold to examine whether an increase in the catalyst concentration would result in higher detection sensitivity in low H_2O_2 concentrations. Each sample contained 4.3 mM papain (two-fold volume), 5 mM TMB, and acetic acid buffer under varying H_2O_2 concentrations identical to previous trials. The strength of colorimetric production, as inferred by the trendline's average rate of change and visually, was weaker than that of our previous trials, and the statistical correlation (R^2 = 0.515) was poor due to background disturbance by papain precipitation. A weak linear correlation existed in the samples of low H_2O_2 concentrations of 0–0.2 mM (**Figure 5**). Increasing the papain concentration by ten-fold produced an

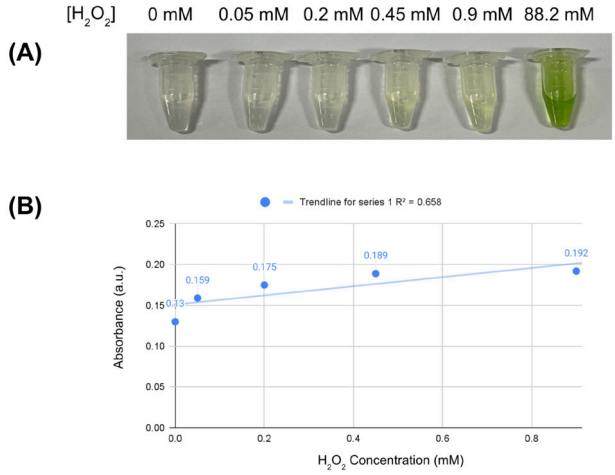

Figure 7. Increasing sample TMB concentration two-fold reduces correlation between H_2O_2 concentration and absorbance at 460 nm. (A) Image of samples under varying H_2O_2 concentrations. **(B)** Linear regression shows a positive correlation between H_2O_2 concentration and absorbance at 460 nm (n=1). Each sample containing 10 µL 4.3 mM papain, 200 µL 5 mM TMB, and 790 µL acetic acid buffer under varying H_2O_2 concentrations (ranging from 0–88.2 mM, 100 µL/sample) was incubated at 40°C for 30 minutes. Coefficient of determination (R^2 = 0.658).

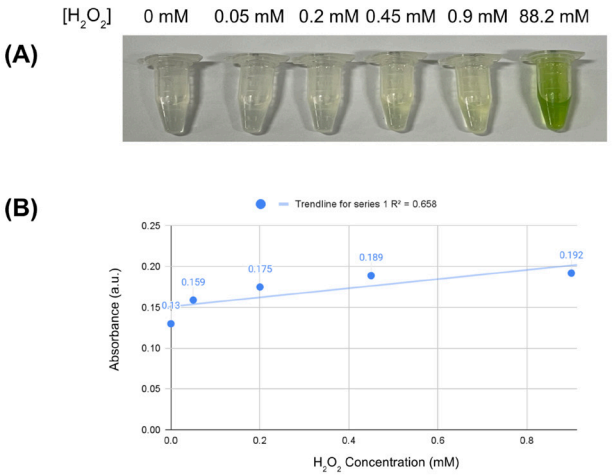

Figure 8. GOx-mediated glucose oxidation and papain-mediated TMB oxidation colorimetrically predict glucose concentrations. (A) Image of samples under varying glucose concentrations. **(B)** Linear regression shows a positive correlation between glucose concentration and absorbance at 460 nm (n=1). Each sample containing 20 µL 1.0 mg/mL GOx and 100 µL PBS under varying glucose concentrations (ranging from 0.0–0.5 mM, 100 µL/sample) incubated at 37°C for 40 minutes was transferred to sample containing 10 µL 4.3 mM papain, 100 µL 5 mM TMB, and 670 µL acetic acid and incubated at 40°C for 20 minutes. Coefficient of determination (R^2 = 0.958).

even weaker colorimetric signal and correlation with a 0.551 coefficient of determination, whereas the disagreement with the trendline was reduced (**Figure 6**). Thus, diverging from the concentration of papain utilized in the previous experiments did not increase the reaction's sensitivity.

Furthermore, to assess whether changes in the substrate concentration increased the detection sensitivity at low H_2O_2 concentrations, we increased the TMB concentration two-fold. Each sample contained 4.3 mM papain, 5 mM TMB (two-fold volume), and acetic acid buffer under varying H_2O_2 concentrations ranging from 0 mM to 88.2 mM, adjusted to the final volume. While the strength of colorimetric signal production was comparable to initial trials, the statistical correlation was comparable with preceding trials, with weak linear correlation (R^2 = 0.658) observed in low to medium H_2O_2 concentrations (**Figure 7**). Hence, manipulating either the substrate or catalyst side of the reaction did not increase measurement sensitivity; the original literature-suggested conditions, with modifications to buffer condition, papain concentration, and incubation time, induced papain-mediated TMB oxidation with robust accuracy.

Finally, we coupled GOx-mediated glucose oxidation and papain-mediated TMB oxidation in vitro to assess their compatibility and correlational relationship. A sample containing GOx-oxidized glucose, containing varying concentrations of glucose (0.0–0.05 mM), was transferred to an optimized medium for papain-mediated TMB oxidation without H2O2. The results indicated a linear correlation between glucose concentrations and absorbance at 460 nm (R^2 = 0.968) and marked production of colorimetric signals (**Figure 8**). Overall, the results suggest that when two reactions are coupled in an optimized setting, colorimetric signals provide a strong predictor of glucose concentrations, validating the papain-based glucometer method's potential use as a novel glucometer method.

Prediction of glucose concentration by this method resulted in a ten-fold increase in detection sensitivity when compared to the previous studies. The point of comparison is the measurement reported by the previous study. In the study, the papain-based glucometer method — with identical conditions to our trials, but with no adjustments proposed herein — was reported to have a reliable detection range (points of a sensitive colorimetric signal production) of 0.1–0.5 mM glucose (14). In this research, we identified a reliable detection range of 0.01–0.05 mM glucose, which is thus ten-fold more sensitive in the detection range.

DISCUSSION

Papain has been proposed as a potent substitute to HRP by quantitatively oxidizing TMB in the presence of H_2O_2; however, the sensitivity of the papain-based glucometer method remains troublesome (14). As a novel glucometer method, our research aimed to couple GOx-mediated glucose oxidation and papain-mediated TMB oxidation to identify an optimized condition catalyzing two reactions.

Foremost, papain-mediated TMB oxidation occurred in the presence of H_2O_2 under the acetic acid buffer (**Figure 2**). The

reaction failed to be catalyzed when incubated in the NaAc-HAc buffer. Production of a significant colorimetric signal at an absorption peak of 460 nm supports the catalysis of papain, which yields OH and oxidizes TMB. The colorimetric signal was produced only after the direct substitution of NaAc-HAc buffer by acetic acid; under NaAc-HAc buffer recommended by previous studies, the colorimetric signal was not produced (14). This suggests that papain exerts peroxidase-like activity and is a possible substitute for HRP. Moreover, the reaction's undisturbed occurrence in a scaled-down experimental setting indicates the reaction's feasibility in various replicable settings.

Second, when incubated under literature-suggested conditions of papain, TMB, and acetic acid buffer, the quantitative strength of chromogen's colorimetric signal selectively predicted H_2O_2 concentrations (**Figure 3**). When the incubation time was increased from 20 to 30 minutes, the absorbance at 460 nm presented linear regression with increasing H_2O_2 concentration with a high coefficient of determination ($R^2 = 0.99$) (Figure 4). The H_2O_2 detection range, when adjusted to the final volume of the sample, was 0.0–-0.9 mM, which yielded a ten-fold sensitivity increase relative to previous studies. Deviating from the literature-suggested optimized conditions did not increase papain sensitivity. Increasing substrate (TMB) and enzyme (papain) conditions neither produced a higher determination of coefficient nor improved papain-mediated TMB oxidation (**Figures 5–7**).

Third, spontaneous catalysis of GOx-mediated glucose oxidation and papain-mediated TMB oxidation is viable. The concentration of glucose and absorbance at 460 nm produced an extremely high correlation ($R^2 = 0.958$) (**Figure 8**). Therefore, the presence of H_2O_2 produced by the GOx-induced oxidation of glucose quantitatively catalyzes the activity of papain. Furthermore, the detection range of glucose was 0.01–-0.05 mM, which is ten-fold sensitive relative to the previous studies. The previous study identified a glucose detection range of 0.1–-0.5 mM, and there exist no scientific disputes regarding its sensitive detection range in the literature (14). Therein, identical experimental conditions to our trials were utilized - with none of the modifications to reaction conditions proposed herein, however. Hence, the scaled-down, optimized conditions and protocols utilized herein produced a ten-fold increase in detection sensitivity in the detection range.

The optimized papain-based glucometer method must differentiate between varying concentrations of glucose for diabetics' daily use. The detection range under which glucose is detected must be wide and minuscule for accurate measurements. The detection range that we have obtained (0.01–-0.05 mM glucose) reflects the minimum glucose concentration detectable when the papain-based glucometer method is optimized on a hydrogel film.

In addition to replicating the viability of this reaction on the 1.5-mL scale, we established three key modifications to the standard protocols: prolonged incubation time (from 20 minutes to 30 minutes), the substitution of NaAc-HAc with acetic acid, and 1000-fold increase in papain concentration (14). This modification prolonged the timespan of the GOx and TMB oxidation reactions, which increased the concentration of the oxidized chromogens that maximally absorb at 460 nm. Samples incubated with NaAc-HAc buffer had a pH of 4.1, whereas samples incubated with acetic acid buffer had a pH of 3.1. It is plausible that the dehydroxylation reaction of TMB is maximized by a strongly acidic medium.

Sodium's inhibitory effects on pepsin-like enzymes have been well understood in the literature (21). Likewise, the treatment of sodium in human gastric juice significantly diminishes the activity of pepsin (21). Thus, besides the consideration of pH in the substitution of NaAc-HAc buffer by acetic acid, the presence of sodium has been suggested as an inhibiting factor to papain, which is a representative pepsin-like enzyme. Hence, all sodium-based compounds were removed from the buffer condition: acetic acid was utilized as an alternative buffer, enabling the catalysis of a sensitive and selective reaction.

A novel glucometer method combining oxidation reactions enables the detection of glucose in varying aqueous conditions with marked sensitivity. This suggests that this method can be utilized to predict glucose concentrations in body fluids secreted by exocrine glands, such as sweat glands. A significant, observable correlation ($R^2 = 0.94$) exists between sweat glucose (SG) and blood glucose (BG). Glucose from interstitial fluid underlying the dermis outwardly diffuses to the skin via a highly vascularized sweat gland, thus reflecting the BG composition (22).

With dramatically increasing DM cases reported worldwide, patient-guided glycemic control (PGC) is necessary to delay the onset or pathogenesis of diabetic complications (1). The invasiveness, high cost, and low accessibility of traditional glucose tests such as DUGT and FBGT impede PGC (10). Hence, when a GOx/papain-mediated glucometer reaction is integrated with emerging hydrogel technology, an accessible and non-invasive glucometer patch can be developed to infer BG from the patient's produced sweat. We are actively investigating this development, and this research constitutes a foundational advance and proof of concept to this development.

There are several limitations to this study. First, the diminished enzymatic activity of GOx and papain may influence the post-incubation analysis of glucose and H_2O_2 sensitivity reported herein. Increasing the storage interval of PBS reduces enzymatic sensitivity. Hence, procedures to evaluate the enzyme unit (U) or preserve the enzyme's functional stability are necessary (23). Second, the background noise of incompletely dissolved supernatant and precipitation may skew the spectrophotometric analysis (24). During analysis of papain-mediated TMB oxidation, minor papain precipitation occurred in most conditions, and TMB was incompletely dissolved in the high TMB treatment. Third, the precise mechanisms of pH dependence on papain activity

are yet to be understood from the performed experiments and existing literature. Fourth, measurements or modifications for factors beyond the reliable detection range were not examined herein.

Beyond papain's observational and scientific modifications, its impediments and costs to industry-wide HRP substitution must be considered (relative to HRP). Due to papain's natural occurrence in C. papaya and its association with food processing industries, papain supply is abundant in the Democratic Republic of Congo, Tanzania, Uganda, and Sri Lanka. However, the commercial papain production process omits the sophisticated processes of drying, cooling, precipitation, and filtration to preserve the enzyme's unit and functional stability, and commercial papain is distributed in a spray-dried form (25). Thus, governmental and institutional interference in the local production agencies must be increased to standardize the papain production process for scientific use.

In conclusion, we examined the feasibility of substituting HRP with papain (reported to display peroxidase-like activity under the presence of H_2O_2) in the DUGT glucometer method under several modifications. We coupled GOx-mediated glucose oxidation and papain-mediated TMB oxidation and analyzed their viability and sensitivity as a novel glucometer method. The optimized conditions catalyzing coupled reactions identified herein agree with the literature (14). Importantly, this optimized condition enabled a ten-fold increase in the detection of glucose via papain compared to previous studies (14). The novel glucometer method can be further integrated with hydrogel technology as a non-invasive glucometer patch measuring BG from SG.

MATERIALS AND METHODS

Chemicals and Instruments

To prepare the stock papain solutions, 0.005 g, 0.05 g, 0.5 g, and 5 g of lyophilized papain powder (Daejung Chemicals, Siheung, South Korea) were vortexed vigorously at 2000 rpm with 100 mL phosphate buffer saline (PBS) to obtain concentrations of 0.0043 mM, 0.043 mM, 0.43 mM, and 4.3mM, respectively. The papain powder and solutions were stored at -20°C.

TMB powder 60 mg (Tokyo Chemical Industry, Tokyo, Japan) was vortexed at 2000 rpm with 100 mL dimethyl sulfoxide (DMSO) to obtain a 5 mM TMB solution. TMB powder and solutions were stored in a light-absent environment at room temperature.

Lyophilized glucose oxidase (GOx) 1.5 mg from Aspergillus niger (Sigma-Aldrich, Missouri, United States) was vortexed (at 2000 rpm) and centrifuged with 1.5 mL PBS to obtain a GOx concentration of 1.0 mg/mL. GOx powder and solutions were stored at -20°C.

H_2O_2 3% solution, with a concentration of 88.2 mM, was diluted by transferring 0.229 µL, 0.918 µL, 2.29 µL, and 4.5918 µL to 50 mL distilled H2O, which yielded diluted concentrations of 5 mM, 20 mM, 45 mM, and 90 mM, respectively. H2O2 solutions were stored at room temperature.

Anhydrous, extra pure D(+)-glucose 18.016 mg (Riedel-de Haen, Seelze, Germany) was mixed with 10 mL PBS to acquire a 10 mM glucose stock solution. The 10 mM stock solution was further PBS-diluted by a dilution factor of 1/100, 2/100, 3/100, 4/100, and 5/100 to obtain glucose concentrations of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM respectively. Glucose powder and solutions were stored at room temperature.

All other reagents (including acetic acid) and scientific workspace and facility (including electronic densimeter) were provided by the University of Suwon Department of Biological Science. NaAc-HAc buffer was prepared by dissolving 0.196 g sodium acetate powder and 2.7356 mL 100% acetic acid in 1 L distilled water. This created a 0.05 M buffer with a pH of 3.6. All reported concentrations of papain, H_2O_2, and glucose are relative to the sample's final volume. H_2O_2 and papain solutions used during previous trials were re-agitated via a vortexer for 60 seconds at 2000 rpm. TMB and acetic acid solutions used during previous trials were thawed at 40°C for 10 minutes.

Papain-Mediated TMB Oxidation

All created samples created by catalyzing papain-mediated TMB oxidation assumed a 1 mL final volume unless otherwise modified. To catalyze papain-mediated TMB oxidation, 10 µL papain solution, 100 µL TMB solution, and 100 µL H_2O_2 solution were added sequentially in 790 µL acetic acid solution and were incubated in 40°C for 20 minutes. Incubated samples were ice-water bathed for 10 minutes to stop the reaction. The incubation time was increased from 20 to 30 minutes to improve the catalyzation activity of papain. Concentrations of papain, TMB, and H_2O_2 were manipulated for the optimization of papain-mediated TMB oxidation.

Experiment 1 included samples of varying papain concentrations (0.0043–4.3 mM) at 3% H_2O_2. Experiments 2 and 3 included samples of varying H_2O_2 concentrations (0–88.2 mM) at 10 µL 4.3 mM papain; the sample of 88.2 mM H_2O_2 functioned as a positive control. Experiment 3 was a replicate of Experiment 2 with increased incubation time (30 minutes) to enhance statistical accuracy. Experiment 4 included samples of varying H_2O_2 concentrations (0–88.2 mM) at 20 µL (two-fold) 4.3 mM papain. Experiment 5 included samples of varying H_2O_2concentrations (0–88.2 mM) at 100 µL (ten-fold) 4.3 mM papain. Experiment 6 included samples of varying H_2O_2 concentrations (0–88.2 mM) at 200 µL (two-fold) TMB. All other concentrations and/or volume were kept constant.

GOx-TMB Coupled Reaction

To perform GOx-mediated glucose oxidation, 100 µL D(+)-glucose solutions of varying concentrations (0.0–0.5 mM) and 20 µL GOx solution were added sequentially to 100 µL PBS. The samples were incubated at 37°C for 40 minutes.

A full volume (220 µL) of incubated samples was transferred to 670 µL acetic acid containing 10 µL 4.3 mM papain and 100 µL TMB. The sample was then incubated for 20 minutes at 40 °C to induce papain-mediated TMB oxidation.

Spectrophotometric Analysis

The samples containing 100 µL 5 mM TMB, 100 µL 88.2 mM H_2O_2, and 790 µL acetic acid under varying papain concentrations (0.0043–4.3 mM, 10 µL/sample) were prepared. After the samples were incubated at 40°C for 20 minutes, a microplate reader (Biotek) was used to find the maximum absorbance from 400–700 nm (with a 20 nm interval). Then, the absorbance at 460 nm was measured to quantify the H_2O_2 concentration of the prepared samples. To quantify the glucose level, the samples containing 20 µL 1.0 mg/mL GOx and 100 µL PBS under varying glucose concentrations (ranging from 0.0–0.5 mM, 100 µL/sample) were prepared and incubated at 37°C for 40 minutes. Then the 100 µL of this sample was mixed with 10 µL 4.3 mM papain, 100 µL 5 mM TMB, and 670 µL acetic acid and incubated at 40°C for 20 minutes. Then, the absorbance at 460 nm was measured to quantify the glucose concentration of the prepared samples.

Statistical Analysis

The coefficient of determination (R^2) between absorbance at 460 nm (in a.u.) and the H_2O_2 concentrations and the corresponding regression line were calculated in Google Sheets. The Prism 8 program was used to analyze the line graph of the absorbance measured with the different wavelengths.

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