

Visibility Enhancement of Laccase-Based Time Temperature Integrator Color by Increasing Opacity

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Abstract Time-temperature integrators (TTIs) based on aqueous enzyme solutions produce transparent colors which lead to difficulty in distinguishing its color change by naked eye. In this present study, this issue has been solved by increasing the opacity of laccase-based TTI without changes in the kinetics (same zero-order reaction) and temperature dependency (similar Arrhenius activation energy values) of the color change. The opacity was increased by introducing TiO₂, latex, BaSO₄, or ZnO, in combination with a hydrocolloid (xanthan gum, acacia gum, pectin, and CMC) into the TTI system. The combination of TiO₂ and xanthan gum was the best. This finding broadened the advantages of laccase-based TTI to more practical uses for consumer convenience.

Keywords Laccase-based TTI, Visibility enhancement, Opacifying agent, Titanium oxide, Hydrocolloid.

Introduction

From the last two decades, the intelligent packaging systems i.e. time-temperature integrators (TTIs) have been used as an important tool in monitoring the quality of foods. TTIs are small labels, which when attached to a food package surface, monitor the time-temperature history of that product by an irreversible visual color change, and this color change of TTIs should reflect the quality of foods¹. Several types of TTIs have been developed based on various operating principles, including mechanical, chemical, electrochemical, enzymatic and microbiological types. Enzymatic TTIs use lipase^{2,3}, laccase⁴, α -amylase⁵, or β -glucosidase⁶.

The lipase enzyme-based commercially available, Vitsab TTI (Vitsab A.B., Malmö, Sweden), shows a pH dependent color change owing to the enzymatic hydrolysis of lipid substrates, and can be used to check the quality assurance during ambient temperature marketing^{5,7}. However, this TTI type is formed by an emulsion system which has considerable drawbacks⁵ such as i) it is practically not possible to obtain a uniform droplet size of emulsion resulting in a high potential variation of the enzymatic reaction rate, and ii) emulsion stability is subjected to low temperature, especially when stored for a long period of time.

A new laccase-based TTI was firstly developed in our

laboratory⁵. This system is considered to be low cost, wide color coding, safe, and green technology. The system color change follows linear kinetics which is simple. Laccase (EC 1.10.3.2) is a metal containing oxidase enzyme that degrades lignin in the absence of lignin peroxidase and manganese peroxidase⁸. The enzyme belongs to the family of multi-copper blue oxidases catalyzing the oxidation of various types of substrates using oxygen as a co-substrate, resulting in its reduction to water⁹. The substrates include a wide variety of organic and inorganic compounds such as mono-, di-, and polyphenols, aminophenols, methoxy phenols, aromatic amines and ascorbate¹⁰. Among these, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) is commonly used as a substrate to create a TTI because it forms a dark blue color when oxidized¹¹.

In an actual practice of product quality monitoring in the real field, the retailers and consumers suggest for more distinguishing color change of TTI by naked eye¹². The reason for the poor visibility of the laccase-based TTI's color change is due to its high transparency. Its base materials are transparent aqueous solutions. In contrast, the lipase enzyme-based TTI was developed using an emulsion base which is opaque. A particular color of an object, as observed by naked eye, results from reflection of visible light waves from the object surface to the eyes. When light falls on an opaque object, some wavelengths are absorbed by the object, and the others are reflected. However, transparent objects additionally allow light to transmit through. Less light intensity is likely to reflect toward eyes. According to the literatures^{13,14}, shorter presentation time is required for the perception of opaque

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surfaces in comparison with that of transparent surfaces. Short time of decision making implies certainty, leading to high reliability.

Several techniques have been studied for the opacity improvement. It is a challenge to identify an appropriate technique which does not consequently cause synergistic or suppressive impacts on the enzyme activities. Viscosity and composition of TTI systems can be influenced by the added opaque and/or viscous materials¹⁵. It was reported that the catalytic activity of enzyme is negatively affected when the viscosity of solution is increased by an addition of viscogenic agents like glycerol, sucrose, trehalose, and salts¹⁶. An increased opacified paint film can successfully be prepared by dispersing titanium dioxide (TiO₂) into film-forming transparent polymeric binders¹³. TiO₂, a white pigment, is the most important and widely used pigment, and accounts for more than 80 percent of global consumption in the coating and plastic industry due to the advantages like high strength, high opacity, low price, and good UV resistance. TiO₂ was also employed as a pigment to provide whiteness and opacity to food products. TiO₂ confers the property of easy dispersibility and enhancing chemical stability¹⁷.

This present study attempted to enhance laccase-based TTI visibility by increasing opacity without interference with the laccase activity. The opacifying materials of interest included TiO₂, latex, barium sulfate, and zinc oxide. These compounds are on the U.S. FDA's list of generally recognized as safe (GRAS), and their applications in minute quantities are considered safe for human consumption. Due to their poor dispersion stability and water solubility, they were scattered in four different hydrocolloid networks created from carboxymethyl cellulose, xanthan gum, acacia gum, and pectin.

Materials and Methods

1. Materials

Laccase, acacia gum (AG), pectin, carboxymethyl cellulose (CMC), titanium oxide (TiO₂), ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) were purchased from Sigma Chemical Co. (USA). Barium sulfate (BaSO₄), zinc oxide (ZnO) were purchased from Samchun Chemical (Korea). The other chemicals such as xanthan gum (XG) and liquid latex paints were obtained from MSC. CO. (Korea).

2. Sample preparation

A substrate solution was prepared with 5 mM ABTS and 0.2% (w/w) opaque reagent solution (TiO₂, latex, BaSO₄, or ZnO) in 100 mM sodium acetate buffer (pH 5.0). To improve the stability of the solution, a 0.225% (w/w) stabilizer (CMC, XG, AG, or pectin) was added. A laccase solution (0.00417 units) was prepared by dissolving the enzyme in 100 mM acetate buffer. A 20 μ L enzyme solution was mixed with 3

mL of substrate solution. Cuvette with the TTI base solution was regarded as a test TTI.

3. Determination of opacity

Opacity of suspension can be well represented by the suspension turbidity¹⁸. The turbidity was measured at 660 nm by spectrophotometer (Optizen 3220V, Mecasys, Daejeon, Korea).

4. Determination of the kinetic and Arrhenius parameters of TTI

The CIE-*L*a*b** values of the samples were measured over storage time by a colorimeter (NE 4000, Nippon Denshoku, Japan) at temperature of 10, 15, and 25°C. The color response, *L** value, was the desired value as having a linear relationship with storage time¹.

$$Y = k \cdot t \quad (1)$$

where *Y* represents the color response value (*L** value), *k* is the reaction rate constant, and *t* is the reaction time.

The activation energy (*E_a*) of the laccase-based TTI was calculated from the Arrhenius equation¹⁹.

$$\ln k = E_a / (R \cdot T) + \ln A \quad (2)$$

where *E_a*, *T*, *R* and *A* are the activation energy (kJ/mol), absolute temperature (K), gas constant (8.314 \times 10⁻³ kJ/mol \times K), and the pre-exponential factor, respectively.

5. Statistical analysis

All data are shown as mean values from three replicate samples. The means were compared using Duncan's multiple range test at a confidential level of 95%. The models were fit to experimental data by linear regression procedures using Microsoft® Excel (2007).

Results and Discussion

1. Impact of opacifying substances

The original laccase-based TTI has a color change in gradient from colorless to transparent green color. Opacifying substances (TiO₂, BaSO₄, ZnO, and latex) were added to the original TTI, but to test opacity, the enzyme was excluded from the TTI base solution to fix the color change. It was found that the opacity was very high (about 88~100% compared with 5% of the original TTI) (Fig. 1). ZnO provided least opacity (*p* < 0.05). The others were similar in the opacity enhancement capacity (*p* < 0.05). However, latex appeared to promptly precipitate once added into the TTI solution. It was reported that pH value has an important impact on the coagulation of polymer latex. At pH 5.0 of the TTI solution,

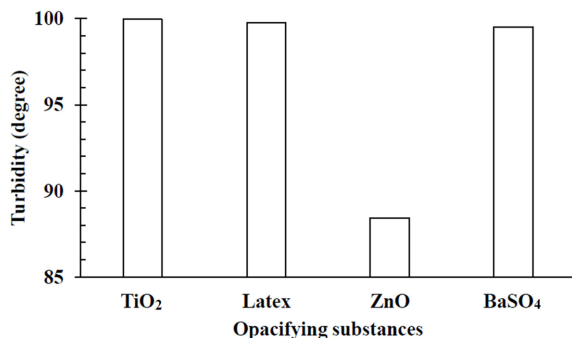


Fig. 1. Turbidity/opacity of TTI with added opacifying substances.

latex was coagulated as predicted (coagulation at pH 4.0-5.8)²⁰. However, the coagulation was more extreme than expected. As a result, ZnO and latex were not used in further investigation.

Regarding the continuous precipitation possible over time, the selected TiO₂ and BaSO₄ were further tested by keeping the TTI base solutions in cuvettes in an upright position. It was found that TiO₂ held constant opacity up to 30 min (Fig. 2). Afterwards, its opacity gradually decreased, showing more precipitation. The dispersion stability can be explained by zeta potential²¹. The presence of zeta potential, surface electrical charge, produces repulsion, increasing dispersion stability. A TiO₂ aqueous suspension was reported to be unstable as its zeta potential is slightly higher than -30 mV at pH 5.0 enough to lead to attraction or precipitation²². A proper range for dispersion stability is higher than 30 mV or lower than -30 mV²². However, a higher pH range is detrimental for the laccase function. At a pH 5.0, the zeta potential is likely to be slightly higher than -30 mV. Therefore, the TiO₂ sample retained suspended for a long period of time before gradually precipitated. For BaSO₄, the opacity decrease was found at an earlier point, and was more intense. The BaSO₄ suspension was homogeneous, and white in color - like the color of milk. It was reported that the zeta potential of BaSO₄ aqueous suspension at pH = 5 was around -5 mV²³. This also implies that the BaSO₄ particle surfaces were slightly negatively charged, and cannot be stable in the suspension. Although TiO₂ and BaSO₄ showed potential for opacity improvement of the TTI, further improvement on their dispersion stability was appreciated.

2. Combination impact of opacifying substances and hydrocolloids

A hydrocolloid (carboxymethyl cellulose (CMC), xanthan gum (XG), acacia gum (AG), or pectin) was additionally mixed into the TiO₂ and BaSO₄ added TTI samples. The turbidity/opacity was observed over storage time and illustrated in Table 1. All the added hydrocolloids helped to enhance the

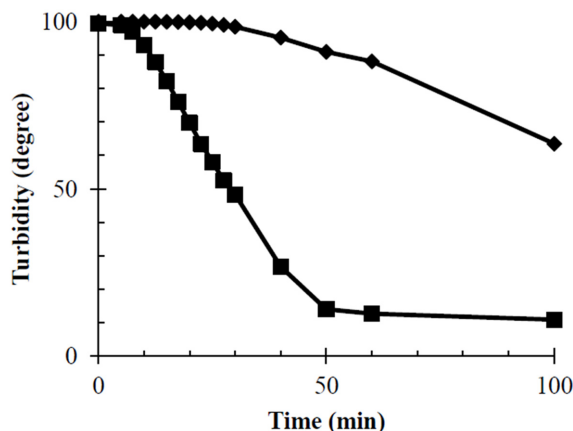


Fig. 2. Turbidity/opacity change of TTI with added opacifying substances over time. \blacklozenge : TiO₂, \blacksquare : BaSO₄.

opacity stability of both TiO₂ and BaSO₄ TTI samples. These new systems extended the constant turbidity periods to longer than those without the hydrocolloids.

The TiO₂-hydrocolloid samples remained well distributed than the BaSO₄-hydrocolloid when the samples kept immobile. Their turbidity was unchanged for 120 min. Centrifugation was further applied to determine the stability after 120 min. In the centrifugation treatment, the stability was distinctively highest when the TiO₂ added TTI was mixed with XG. XG is an anionic gum, forms very viscous solutions at low concentrations, and very stable in a wide range of pH values^{18,23,24}. Such high viscosity of XG helps to hold and disperse the TiO₂ particles. In addition, at pH = 5, XG molecules are a polyelectrolyte, highly dissociated and defined by a similar structure (charge and molecular size) for pH > 3²⁵. The carbonyl groups of XG molecules are likely to adsorb any present positively charged sites^{26,27}, making the TiO₂-XG TTI more highly negative (lower than -30 mV). Such high repulsive forces between the negatively charged TiO₂ and xanthan surfaces further enhanced the stability of the suspension system. In addition, XG has a shear-thinning behavior²⁸. During the centrifugal sedimentation, the particles (TiO₂) move through the XG suspension with shearing forces^{29,30}. The viscosity of shear thinning fluid reduces with sedimentation velocity³¹. As the XG viscosity reduced, the TiO₂-XG TTI was still very stable. This implies that the stability of such suspension system is more strongly associated with the electrostatic repulsion than viscosity. This evidence is very important. During storage and distribution, mechanical damages to the products can occur. TTIs, therefore, must withstand forces.

The BaSO₄-hydrocolloid TTI was less stable than the TiO₂-hydrocolloid. This is possibly related to the total negative charge on the metal oxide particle surfaces. As stated, the BaSO₄ surfaces were negatively charged but in a much smaller

Table 1. Turbidity changes over storage time of TTI with added with TiO₂ and BaSO₄ in combination with a stabilizer

Sedimentation time (min)	TiO ₂				BaSO ₄			
	XG ^b	AG ^b	Pectin	CMC ^b	XG	AG	Pectin	CMC
0	99.97	99.97	99.95	99.97	99.65	99.42	99.38	99.43
5	99.97	99.97	99.95	99.97	99.64	99.36	99.34	99.40
10	99.97	99.97	99.95	99.97	99.64	99.20	99.32	99.35
15	99.97	99.97	99.95	99.97	99.63	98.62	99.29	99.19
20	99.97	99.96	99.95	99.97	99.62	97.07	99.20	98.77
25	99.97	99.96	99.95	99.97	99.62	93.78	99.03	97.89
30	99.97	99.96	99.95	99.97	99.62	89.45	98.76	96.51
40	99.97	99.96	99.95	99.97	99.61	75.81	97.60	90.84
50	99.97	99.95	99.95	99.97	99.60	57.02	95.13	81.43
60	99.97	99.95	99.95	99.97	99.60	43.45	90.94	70.13
70	99.97	99.95	99.95	99.97	99.59	36.67	85.24	54.72
80	99.97	99.94	99.95	99.97	99.59	33.90	78.36	40.00
90	99.97	99.94	99.95	99.97	99.58	34.35	70.16	27.54
100	99.97	99.94	99.95	99.97	99.58	34.19	62.78	21.15
110	99.97	99.94	99.95	99.97	99.58	34.14	53.64	18.47
120	99.97	99.94	99.95	99.97	99.58	33.81	44.34	17.88
Centrifuged ^a	99.96	13.35	0	35.68	75.62	18.62	0	0

^aThe samples were centrifuged at 4,200xg for 15 min.

^bXG: xanthan gum, AG: acacia gum, CMC: carboxymethyl cellulose

degree than that of the TiO₂. As a result, the combination of the BaSO₄ with the hydrocolloids is likely to result in less negatively repulsive forces and less stability of the suspension.

CMC is an anionic polymer which is capable of forming very viscous solutions but lower than XG at the same concentrations³²). CMC has negative charges with a zeta potential value slightly above -30 mV³³). With such high viscosity and electrostatic repulsion, it can well disperse TiO₂ but not BaSO₄. Like xanthan, it has a shear-thinning behavior³⁴). After the centrifugation, more than a half of TiO₂ and all BaSO₄ were precipitated out. Actually, the native CMC had similar net surface charge to the native XG. So the lower metal suspension stability is possibly due to the lower viscosity of CMC than that of XG.

AG has relatively lowest viscosity compared to the other used hydrocolloids³²) and has maximum viscosity at pH 5.0-5.5³⁵). However, at pH 5.0 it is negatively charged with the high zeta potential value near -40 mV³⁶). Such high electrostatic repulsion can stabilize the TiO₂ suspension, but not the BaSO₄, still. After centrifugation, both metals were largely precipitated out. This is possibly associated with its shear-thinning behavior³⁷). The more negatively charged surface of native AG than native XG had not made the TiO₂ and BaSO₄ particles more stably suspended. This implies that the combination of the metals and XG potentially led to stronger electrostatic repulsion, or the high viscosity of XG had additionally important impacts on the suspension stability.

Low methoxyl pectin forms gel network in a presence of bivalent ions (e.g. Ti²⁺, Ba²⁺, Ca²⁺, Sr²⁺)^{38,39}). The result suggests that the Ti²⁺ ions are better held by the pectin network than the Ba²⁺. However, the holding strength was not comparable to the centrifugal forces. The Ti²⁺ and Ba²⁺ ions were forced out from the network. This is possibly due to the fact that the divalent pectin networks have shear-thinning behaviors^{40,41}).

3. TTI performance

The color change over time is a most important performance of TTI, demonstrated in Fig. 3. It is clear that the color visibility of the TiO₂-XG added TTI is higher than the original laccase-based TTI (no TiO₂-XG) due to increasing in opacity. The gradual change of color from opaque white (milky) to opaque green is more easily noticeable by naked eye than from transparent colorless (water-like) to transparent green.

The new improved laccase-based TTI was further evaluated for the kinetic color change and related temperature dependent parameters. The kinetic color change by the L^* value of the new TTI was found to be a zero-order reaction at all the examined temperatures ($R^2 > 0.98$) (Table 2). The predicted data were in an excellent agreement with the experimental (Fig. 4). Similarly, the color change response of the original laccase-based TTI showed a zero-order reaction as well⁵).

The temperature dependence of the color change rate of the new improved laccase-based TTI was well described by the



Fig. 3. The color changes over time (from left to right) of the original TTI (top row) and new improved TTI (bottom row).

Table 2. The kinetic equations at different temperatures and Arrhenius parameters for the new improved TTI color (L^* value) changes

Temp. (°C)	Kinetic equations ^a	R ² ^b	Ea ^c (kJ/mol)	R ²
10	$Y = -0.179 \cdot t$	0.9912	52.37	0.9941
15	$Y = -0.243 \cdot t$	0.9873		
25	$Y = -0.540 \cdot t$	0.9819		

^aY: L^* value, t: time (min).

^bCoefficient of determination.

^cArrhenius activation energy

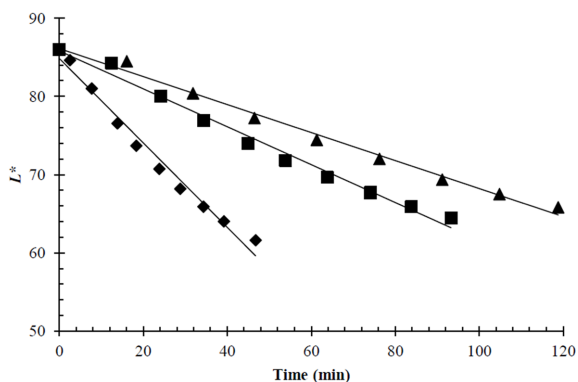


Fig. 4. The plot of experimental and predicted TTI color response (L^* value) vs. time at different temperatures. ◆: 25°C ■: 15°C, ▲: 10°C.

Arrhenius equation. Interestingly, the activation energy (E_a) was equal to 52.37 kJ/mol which is almost the same as that of the original laccase-based TTI ($E_a = 55.48$ kJ/mol)⁵. This similarity suggests that the new improved laccase-based TTI can be used interchangeably with the original one. An additional presence of TiO_2 and XG do not significantly interfere with the laccase-substrate interaction. The laccase and substrate remain reacting in response to time and temperature in such new environment with higher viscosity

and electrostatic repulsion.

Potential applications of the new TTI are diverse. The original laccase-based TTI had been applied in order to monitor the quality of beef, and found a high accuracy in quality evaluation by the TTI when compared the predicted qualities with those of the real beef¹². This implies that the new TTI can be a good alternative for the beef quality monitoring. Also, the E_a value of this new laccase-based TTI is similar to the E_a value (50.2 kJ/mol) of the commercial lipase enzymatic type TTI C2-15d (Vitsab AB, Sweden)³. The new TTI is then likely to be a good competitive alternative of this commercial one. Other food product qualities with the similar E_a value (52.37 kJ/mol) include fruit and vegetable respiration rates ($E_a = 29-79$ kJ/mol)⁴².

Conclusion

An original laccase-based TTI with transparent colors has been successfully modified into the one with opaque colors, which is more visibly distinguished. The original one was improved in the opacity when added with TiO_2 and $BaSO_4$. ZnO and latex were extremely precipitated; not proper for the opacification of the original system. The TiO_2 and $BaSO_4$ suspension can be further enhanced by an addition of xanthan gum. Other hydrocolloids (pectin, acacia gum, and CMC) supported the suspension stability but not for a long period of time. However, the combination of TiO_2 and xanthan gum provided most stable suspension, especially when centrifugal forces were imposed. This implies that the opacity of the TiO_2 -XG added laccase-based TTI is durable against mechanical damages at least for some degrees. In addition, this new laccase-based TTI has been verified to have the same kinetic zero order reaction and activation energy value as those of the original one. They are likely to be used interchangeably. However, the new one possibly provides better visibility to customers as its opacity is far higher. It is potential for usages in the actual field than the original.

Acknowledgement

This work was supported by the Dongguk University Research Fund of 2020-21.

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투고: 2021.08.03 / 심사완료: 2021.08.18 / 게재확정: 2021.08.23