J Korean Soc Appl Biol Chem (2013) 56, 419–425
DOI 10.1007/s13765-013-3093-x

ORIGINAL ARTICLE

Application of Mixture Rule to Determine Arrhenius Activation Energy of Time Temperature Integrator Using Mixture of Laccase from *Pleurotus ostreatus* and PEGylated Laccase from *Trametes versicolor*

Tae Jin Kim · Dong Yeol Choi · Ki-Hong Yoon · Keehyuk Kim · Seung Ju Lee

Received: 19 April 2013 / Accepted: 1 July 2013 / Published Online: 31 August 2013

Abstract Two isoenzyme mixture system was developed to freely adjust the Arrhenius activation energy (*E*a), which is one of the most important parameters to design a reliable time temperature integrators (TTIs). We hypothesized that the apparent *E*a of a mixture of two isozymes would be expressed in a simple linear relationship with the ratio of the mixture, although it could be expressed with a rather complicate equation. To prove our hypothesis, laccase from *Pleurotus ostreatus* (*E*a =27.06 kJ/mol) and PEGylated laccase from *Trametes versicolor* (*E*a =50.35 kJ/mol) were used to prepare enzyme mixtures with ratios of 0, 0.25, 0.5, 0.75, and 1.0. The enzyme activity was determined by the increase of absorbance of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) incubated at 5–30°C, pH 5.0, and *E*a for each enzyme mixture was determined to be in the range of 27.06–50.35 kJ/mol. Relationship between *E*a and a ratio of the enzyme mixture not only turned out to be linear, but also was well fitted to the linear mixture rule. This newly found linear dependency is much simpler than kinetically derived one, presumably because microscopic reaction paths and thermodynamic parameters were combined and cancelled out, resulting in linearity. This finding is important in that a mixture of two enzymes with a proper ratio determined from the simple linear mixture rule can customize *E*a of an enzymatic TTI. This easier and convenient method can offer a practical and reliable way to adjust *E*a of an enzymatic TTI on demand.

T. J. Kim · D. Y. Choi · S. J. Lee (✉)
Department of Food Science and Biotechnology, Dongguk University-Seoul, Seoul 100-715, Republic of Korea
E-mail: Lseungju@dongguk.edu

K. -H. Yoon · K. Kim
Department of Culinary Nutrition, Woosong University, Daejeon 300-718, Republic of Korea

Keywords arrhenius activation energy · simple mixture rule · enzyme mixture system · laccase · time temperature integrator · temperature dependence

Introduction

Recently, time temperature integrators (TTIs) have drawn interests of scientists and engineers, whose main purpose is to study the time-temperature history in environmental thermal pollution (Zhao et al., 2013), food distribution, and storage (Taoukis and Labuza, 1989). TTI is an attachable device or smart label that shows the accumulated time-temperature history of a site or a product in a non-penetrating manner. TTIs are commonly used on food, pharmaceutical, and medical products to indicate exposure to excessive temperature. A critical aspect of TTI design is a good match of *E*a between TTI and target component, because rate of reaction taken place in a TTI device must correlate with the rate of changes, degradation or spoilage of a target material upon increase and decrease of temperature (i.e., $\nu = Ae^{Ea/RT}$, where ν is the rate of reaction (s^{-1}), A is the pre-exponential factor (s^{-1}), *E*a is the activation energy (kJ/mol), R is the ideal gas constant, and T is the absolute temperature (K).

There have been many studies on the applications of TTI to food safety and quality predictions (Taoukis and Labuza, 1989; Giannakourou et al., 2001; Vaikousi et al., 2009). To date, several attempts have been made to apply TTI to monitor the time temperature history at any stage of distribution for fish, dairy, and meat products, as well as fruits and vegetables (Giannakourou and Taoukis, 2003; Guiavarc'h et al., 2003; Giannakourou et al., 2005; Vaikousi et al., 2009). Taoukis et al. (1999) showed that TTI could be used to monitor the shelf life of chilled fish under dynamic storage conditions with predictive modeling, followed by evaluation

in the field. Several types of TTIs have been developed based on various operating principles, including mechanical, chemical, electrochemical, enzymatic, and microbiological types. Enzymatic TTIs use lipase, α-amylase, β-glucosidase or laccase (Kim et al., 2012a). Laccase TTI is promising in terms of flexibility of substrate selection, linear kinetics, and simple composition.

Recently, our lab has developed the new enzymatic TTI that uses laccase (Kim et al., 2012a). Because laccase follows simple discoloration kinetics (Huber and Carré, 2012), the color response to time-temperature variation was linearly proportional to the rate of color change, allowing direct and intuitive determination of the remaining shelf-life of food quality losses (Park et al., 2013). However, the major drawback of enzymatic TTI for monitoring chilled food shelf life is not only the narrow range of *E*a, but also the *E*a value is by no means comparable to that of food spoilage and deterioration (Tsoka et al., 1998; Bobelyn et al., 2006; Kim et al., 2012a; Kim et al., 2012b). Taoukis (2001) suggested that any TTI device with *E*a within 25 kJ/mol from a food quality factor of interest could be acceptable to ensure accuracy of an enzyme TTI for prediction of the remaining shelf-life of food. Therefore, special methods on flexible *E*a adjustment of laccase must be invented for the laccase TTI to accurately reflect the thermal exposed history of a given food quality factor, whose *E*a varies widely from 42–251 kJ/mol (Yan et al., 2008). Yan et al. (2008) reported that *E*a varied with α-amylase contents in the range of 102–114 kJ/mol, yet it was not sufficient enough to cover a variety of foods' *E*a, which spans 42–251 kJ/mol. Park et al. (2013) recently reported that sodium azide (NaN_3), a reversible non-competitive type inhibitor against laccase, could provide a successful method to adjust a wide range of *E*a (63–112 kJ/mol). However, the addition of NaN_3 may not be safe for both the environment and human health due to its toxicity, and therefore, should be avoided, if possible.

Here we suggest that it could be possible to invent an environmentally safe enzymatic TTI system by mixing two isozymes. This concept is interesting in that if we mix two isozymes (namely, enzymes which catalyze the same substrate, and yet have different *E*a's) for a ratio determined by a simple linear equation, and if this mixture provides a desired *E*a of interest with a linearly dependent manner, we could develop a fairly new method to customize an enzymatic TTI at will. In fact, Casal et al. (2003a) reported that the use of different *E*a values (i.e., 41.8 and 75.1 kJ/mol) of β-hexosamidase isoenzymes was successful for performing diagnosis and heterozygote screening of GM2 gangliosidoses. Similar approach was applied to determine the B1 variant of Hex in mononuclear and polymorphonuclear leukocyte populations (Casal et al., 2003b). In addition, seasonal variations of lactate dehydrogenases (LDH) from white muscle and liver of Norwegian coastal cod for thermal acclimation was determined by measurements of *E*a changes for pooled LDH isozymes from each tissue (Zakhartsev et al., 2004).

To elucidate the possible use of a simple linear relationship between the ratio of two enzymes and *E*a changes, we firstly hypothesized that three practical approaches described earlier would hold true, and then, tried to prove it through experiments. As for a model for isozymes, the PEGylated laccase (50.35 kJ/mol) and native laccase from *P. ostreatus* (27.06 kJ/mol) were chosen and examined whether or not the mixture rule could predict an *E*a of a mixture of two different laccases in practice.

Thus, objectives of the present study were to investigate whether the linear mixture rule can predict the variation of *E*a of the enzyme mixture system, and the prediction capability of TTIs was evaluated to ensure that the customized TTI shows a consistent color (absorbance) change at a fixed freshness, regardless of the time-temperature experiences.

Materials and Methods

Materials. Two types of laccases (EC 1.10.3.2) either purified from *P. ostreatus* (Pos-laccase) or from *T. versicolor* were purchased from Sigma Chemical Co. (USA). Sodium acetate, acetic acid, [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)], and bovine serum albumin (BSA) were from Sigma Chemical Co. Methoxypolyethylene glycol activated with cyanuric chloride (average mol wt 5000) was obtained from Sigma Chemical Co. and used for chemical modification (PEGylation) of laccase from *T. versicolor*.

Laccase activity assay. Laccase solution was prepared in 10 μg BSA and 50 mM sodium acetate buffer, pH 5.0. The standard assay condition was as follows (Kunamneni et al., 2008): The standard assay solution was composed of 5 mM ABTS in 100 mM sodium acetate buffer, pH 5.0. A total of 3.0 mL of the reaction mixture was pre-equilibrated at 30°C, and then 20 μL of the enzyme solution was added, followed by the measurement of ABTS oxidation based on the increase of absorbance at 430 nm ($\varepsilon_{430\,nm}$ =36,000 M^{-1} cm^{-1}). One unit of the laccase activity corresponds to the oxidation of 1 μmol ABTS per minute under these conditions.

Preparation of PEGylated laccase from *Trametes versicolor*. The chemical modification of *T. versicolor* was carried out as described by López-Cruz et al. (2006) and Kim et al. (2012a). A mixture of the laccase and cyanuric chloride-activated monomethoxypolyethylene glycol (mol wt 5000) at a ratio of 1:50 was prepared in a solution of borate buffer (pH 10.0). This reaction mixture was placed in a 25°C water bath for 6 h to complete the reaction. Subsequently, a 9-fold volume of the cold 100 mM sodium acetate buffer (pH 5.0) was immediately added to stop the reaction. The PEGylated laccase was concentrated by ultrafiltration.

Determination of *E*a for a mixture of native and PEGylated laccase. An enzyme solution for higher *E*a was prepared with the PEGylated laccase (0.0015 unit/mL) dissolved in 1.0 μg/mL BSA and 100 mM sodium acetate (pH 5.0). Another enzyme solution (native laccase, Pos-laccase) for lower Ea was prepared with laccase from *P. ostreatus* (0.0015 unit/mL) dissolved in 1.0 μg/mL BSA and 100 mM sodium acetate (pH 5.0). Enzyme mixtures in

the range of 0 to 100% were prepared by mixing each enzyme solution. Mixing ratios of two enzymes were noted as 0, 25, 50, 75, and 100%. For example, 0% denotes Pos-laccase only; 50%, an equal amount of mixture with Pos-laccase and PEGylated laccase; 100%, PEGylated laccase only. A substrate solution was composed of 5 mM ABTS and 100 mM sodium acetate buffer (pH 5.0). Assay was initiated by mixing 20 μL of enzyme mixture with the substrate solution (3 mL) already thermally equilibrated at 5, 10, 15, 20, 25, and 30°C.

The laccase-based TTI response was reported to follow a zero-order reaction (Kim et al., 2012a).

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

where X is the absorbance measured at 430 nm, k is the reaction rate constant (1/h), and t is the reaction time (h). The reaction rate constant was determined from the slope of the line created by plotting the reaction time against absorbance under isothermal conditions. The Arrhenius activation energy was estimated by the Arrhenius equation expressed in logarithmic terms Eq. (2).

$$\ln k = \ln A + \{(-Ea)/R\} \cdot (1/T) \quad (2)$$

where k is the rate constant (1/h), A is the pre-exponential factor (1/h), Ea is the activation energy (kJ/mol), R is the ideal gas constant ($8.314 \times 1/10^3$ kJ/K · mol), and T is the absolute temperature (K). The slope ($-Ea/R$) and the intercept ($\ln A$) were obtained by linear regression.

Based on the kinetic modeling we can theoretically calculate the rate of reaction, and a combined Ea of the enzyme mixture system as well. In enzyme kinetics, it has been assumed that if a reaction occur by two distinct enzymes through unique reaction path specific to each enzyme, and both enzymes are mutually independent and do not interact during the reaction.

$$E1 + S \leftrightarrow E1 + P \quad (3)$$
$$E2 + S \leftrightarrow E2 + P \quad (4)$$

where $E1$ and $E2$ are two different isozymes; S is a substrate for $E1$ and $E2$; P is the single product. Thus, the rate of reaction in a mixture of S, $E1$, and $E2$ would be as follows.

$$k_{E1} = V_{\max E1}[S]/(K_{mE1} + [S]) \quad (5)$$
$$k_{E2} = V_{\max E2}[S]/(K_{mE2} + [S]) \quad (6)$$
$$k_{mix} = \varphi_{(E1)} k_{E1} + \varphi_{(E2)} k_{E2} \quad (7)$$

where k, $V_{\max}$, and K_m are the reaction rate, maximum rate, and Michaelis constant, respectively. φ is the volume ratio, i.e., $\varphi_{(E1)} = [E1]/([E1]+[E2])$, and $\varphi_{(E2)} = [E2]/([E1]+[E2])$. In practice, the concentration of a substrate was set to the range of the zero-order kinetics, i.e., $[S] >> Km$, above equation can be simplified into Eq. (8).

$$k_{mix} = \varphi_{(E1)} k_{E1} + \varphi_{(E2)} k_{E2} = \varphi_{(E1)} V_{\max E1} + \varphi_{(E2)} V_{\max E2} \quad (8)$$

Substituting the Arrhenius equations into Eq. (8),

$$k_{mix} = \varphi_{(E1)} k_{E1} + \varphi_{(E2)} k_{E2} = \varphi_{(E1)} A_{E1} \exp(-Ea_{E1}/RT) + \varphi_{(E2)} A_{E2} \exp(-Ea_{E2}/RT) \quad (9)$$

where A_{E1}, A_{E2} and Ea_{E1} and Ea_{E2} are Arrhenius constants and activation energies of $E1$ and $E2$ respectively. Although additivity of Arrhenius activation energies in binary enzyme system appeared to be true in Eq. (9), the resulting equation was so complex that Ea of the mixture could not be determined according to the linear relation between Ea of two enzymes mentioned above.

Statistical analysis and validation of observed and predicted values from mixture rules. The goodness-of-fit of mixture rules was evaluated by the bias factor (B_f, Eq. (10)) and the accuracy factor (A_f, Eq. (11)). In all cases, the B_f and A_f values were close to unity, which indicated a good fit between the observations and the predictions. Dalgaard and Jorgensen (1998) reported these two factors were valuable tools for the evaluation of predictive models.

B_f is an estimate of the extent of under- or over-prediction by the model, because it gives the structural deviations, but cannot indicate the average accuracy of the estimates. A_f is an estimate of the averaged distance between each point and the line of equivalence as a measure of how close the predictions are to the observations (Ross, 1996).

$$B_f = 10^{(\Sigma_{i=1}^{n} \log(obs/pred)/n)} \quad (10)$$

$$A_f = 10^{(\Sigma_{i=1}^{n} |\log(obs/pred)|/n)} \quad (11)$$

where n is the number of observations, obs is the observed value, and pred is the predicted value. Kinetic and Arrhenius parameters were estimated by linear regression and other basic statistical measures: mean, standard deviation, and coefficient of variance (CV) were calculated by using MS Excel.

Performance of prototype laccase TTI containing enzyme mixture under dynamic storage condition. For the dynamic storage experiment, non-isothermal temperature storage profiles for 3 h at 5°C, 3 h at 10°C, and 3 h at 15°C, was used, and this process was repeated 6.5 times. A laccase-based prototype TTI was composed of two small separate chambers on a plastic sticker film: one chamber contained an enzyme solution and another, a substrate solution. To initiate the reaction, both chambers were pressed with finger tip, forcing to open the sealed channel, which is connected to both chambers, and as a result, enzyme and substrate solutions were mixed together through this channel.

An enzyme solution for higher Ea was prepared with the PEGylated laccase (0.0015 unit/mL) dissolved in 1.0 μg/mL BSA and 100 mM sodium acetate (pH 5.0). Another enzyme solution for lower Ea was prepared with Pos-laccase from *P. ostreatus* (0.0015 unit/mL) dissolved in 1.0 μg/mL BSA, 100 mM sodium acetate (pH 5.0). The unmixed type of prototype TTI for the PEGylated laccase only or for a Pos-laccase only was performed and validated its performance (data not shown). To examine whether a mixed laccase TTI prototype under dynamic temperature changes would work as predicted by a simple mixture rule, a typical mixed laccase TTI (0.0081 unit/mL, $\varphi_{(E2)}=0.5$) was prepared. About 40 prototype TTIs with the same composition were

prepared and initiated at the same time for the dynamic storage experiment. At each time interval, two pieces were taken out of stored TTIs, all of which were placed on the same shelf in the programmable incubator (HST-103SP, HANBAEK ST, Korea). A reaction mixture of each prototype TTI sample was collected, and the absorbance at 430 nm was measured at each given time intervals specified in Fig. 4. All measures were duplicated. These measurements were compared and analyzed with the predicted value to check for validation.

Results and Discussion

Kinetic parameters under isothermal conditions. As described in the Materials and Methods, the same enzyme unit of Pos-laccase originated from *P. ostreatus* (*E1*) and the activated-PEG modified laccase, *T. versicolor* (*E2*), were mixed, and initial velocities were measured. At 30ºC, basic kinetic parameters (V_{max}, K_m) were determined for three different enzyme mixtures, i.e., $\varphi_{(E2)}$ = 0.0, 0.5, and 1.0.

Under the specified experimental condition, K_m value of Pos-laccase showed 0.06 mM and that of PEGylated laccase was 0.21 mM. It is noticeable that K_m value of the equal enzyme mixture ($\varphi_{(E2)}$ =0.5) was 0.14 mM, which is about an average of K_m values for both native and PEGylated laccase whereas the Vmax values were estimated to be 0.421, 0.411 and 0.416 Abs/min for $\varphi_{(E2)}$ = 0.0, 0.5, and 1.0, respectively.

The enzyme reaction progress curves were shown to be all linear for temperature ranges tested (Fig. 1A and 1C), representing a typical behavior of zero-order reaction. This tendency still holds true even when two enzymes were mixed half and half, i.e., $\varphi_{(E2)}$ =0.5 (Fig. 1B). To make sure that all reactions were performed under the zero-order kinetics, the substrate concentration should be kept at 5 mM ABTS, which is at least 25 times higher than K_m values for all enzyme solutions used. Under this condition, rate constants estimated at each temperature were assumed to be V_{max}. Rate constants of ABTS oxidation reaction with the addition of each enzyme only or enzyme mixtures of given ratios of two enzymes were carefully estimated (Table 1). Reaction rate constants increased with temperature under all experimental conditions.

Temperature dependence of the enzyme mixtures and Arrhenius activation energies. Eq. (2) was applied to fit the data collected from TTIs with varied ratios of the two enzymes at different temperatures (Fig. 2 shows a typical data and fits). Estimated rates are listed in Table 1.

Table 1 shows the rate constants, *k*, and the standard deviation (SD), of six TTI's, the highest SD <0.0116. This highly accurate 0 order reaction fitting could enable the laccase-based TTI prototype to more accurately trace the history of temperature abused environment in which the food products might undergo. To estimate the Arrhenius activation energy (*E*a), ln *k* values were

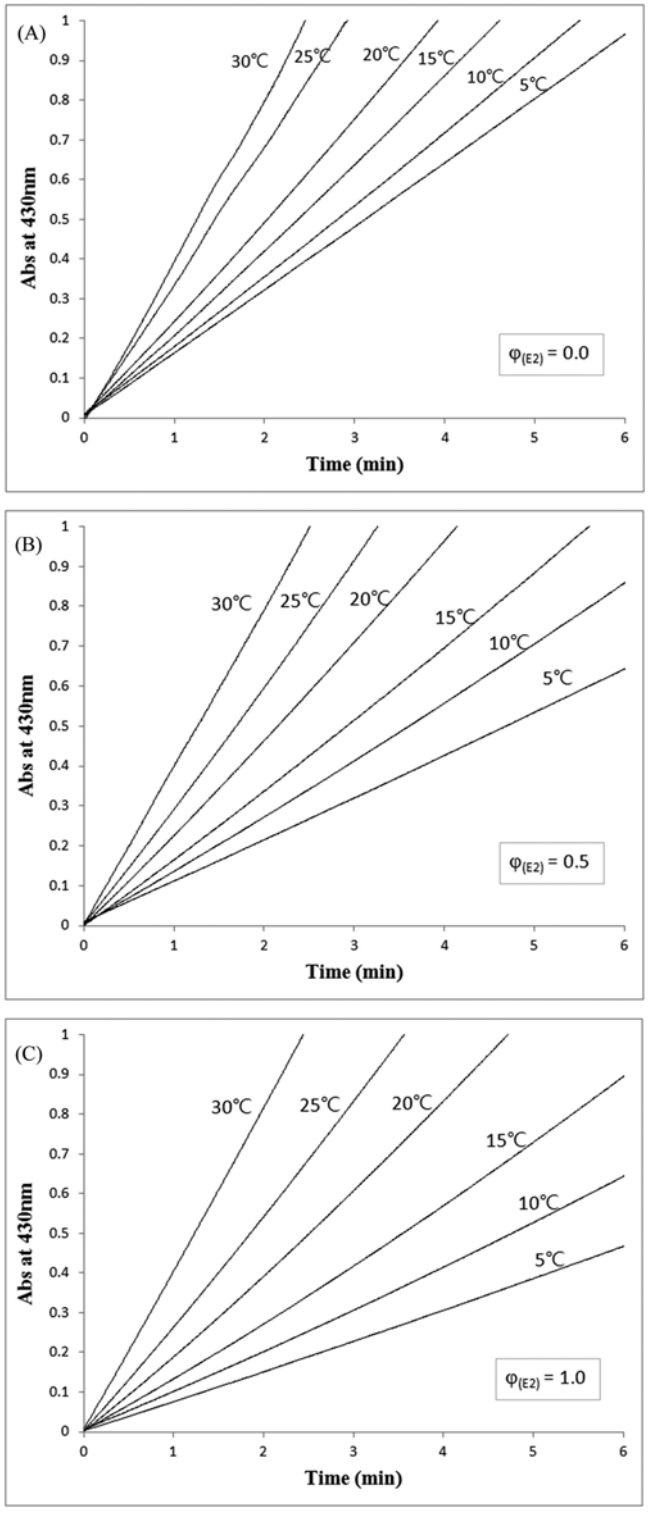

Fig. 1 The zero-order reaction progress curves at 5, 10, 15, 20, 25, and 30ºC for the enzyme mixture of Pos- and PEGylated- laccase in different ratios. (A): $\varphi_{(E2)}$=0, (B): $\varphi_{(E2)}$=0.5, (C): $\varphi_{(E2)}$=1.0.

Table 1 The reaction rate constants (k) and Arrhenius activation energy (Ea) of TTIs of the enzyme mixture of Pos- and PEGylated-laccase in different ratios

TTI	Temperature (°C)	Reaction rate constant±SD[b] (1/min)	Ea±SE[c] ($R^{2,d}$) (kJ/mol)
$\varphi_{(E2)}$[a]=0.0	5	0.1544±0.0045	27.06±0.58 (0.9931)
	10	0.1894±0.0054	
	15	0.2281±0.0083	
	20	0.2769±0.0078	
	25	0.3421±0.0024	
	30	0.4027±0.0065	
$\varphi_{(E2)}$=0.25	5	0.1257±0.0040	33.46±0.67 (0.9956)
	10	0.1619±0.0038	
	15	0.2099±0.0069	
	20	0.2590±0.0037	
	25	0.3381±0.0050	
	30	0.4123±0.0094	
$\varphi_{(E2)}$=0.5	5	0.1027±0.0022	38.40±0.52 (0.9971)
	10	0.1398±0.0040	
	15	0.1849±0.0037	
	20	0.2466±0.0071	
	25	0.3164±0.0051	
	30	0.4040±0.0089	
$\varphi_{(E2)}$=0.75	5	0.0088±0.0033	43.14±0.56 (0.9962)
	10	0.1214±0.0032	
	15	0.1614±0.0062	
	20	0.2276±0.0025	
	25	0.3031±0.0116	
	30	0.4077±0.0051	
$\varphi_{(E2)}$=1.0	5	0.0690±0.0027	50.35±0.56 (0.9979)
	10	0.0990±0.0023	
	15	0.1431±0.0027	
	20	0.2041±0.0042	
	25	0.2971±0.0097	
	30	0.4111±0.0030	

[a]$\varphi_{(E2)}$ denotes the fraction of PEGylated laccase ($E2$). $\varphi_{(E2)}$=0.5 indicates a 1:1 mixture of Pos-laccase and PEGylated laccase in terms of enzyme unit.
[b,c,d]SD, SE, and R^2 represent standard deviation, standard error, and regression coefficient, respectively.

plotted against 1/T (Fig. 2). Ea values for five TTI's ranged from 27.06 to 50.35 kJ/mol (Table 1).

Because TTI contained higher ratio of PEGylated laccase, Ea of TTI increased from 27.06 to 50.35 kJ/mol. As expected, activation energies with varied ratio of enzyme mixture TTIs decreased linearity. This is an important finding in that Ea, in spite that several microscopic differences in enzyme reaction mechanism and Arrhenius constants between two enzymes appeared to follow the linear mixture relationship.

Analysis of the relationship between *Ea* and enzyme fraction (φ). Fig. 3 represents the relationship between Arrhenius activation energies (Ea) and the ratio of the enzyme mixtures of Pos- and PEGylated-laccase ($\varphi_{(E2)}$).

As described by Kim and Lee (2009), the mathematical rule of mixture were summarized as follows,

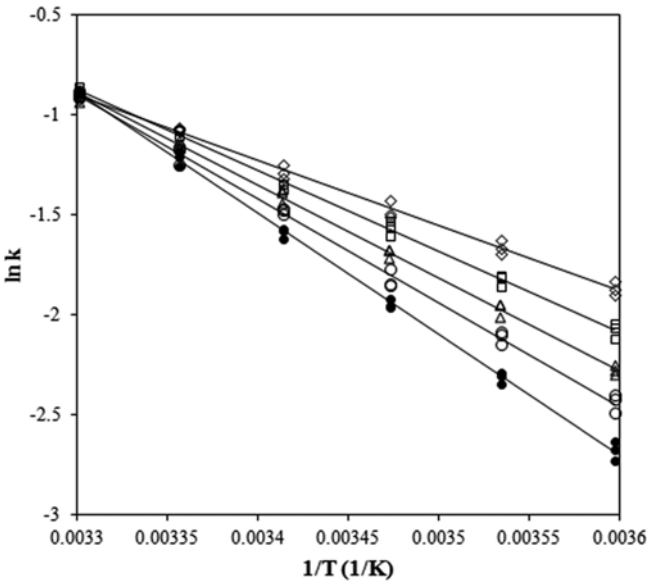

Fig. 2 Plots of ln k vs. 1/T for the enzyme mixture of Pos- and PEGylated-laccase in different ratios. ◇: $\varphi_{(E2)}$=0, □: $\varphi_{(E2)}$=0.25, △: $\varphi_{(E2)}$=0.5, ○: $\varphi_{(E2)}$=0.75, ●: $\varphi_{(E2)}$=1.0.

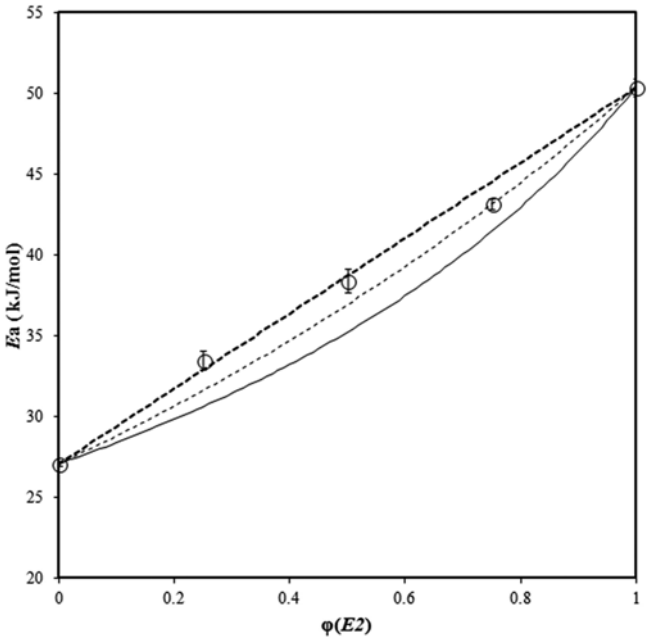

Fig. 3 Relationship between the Arrhenius activation energy (Ea) and the ratio ($\varphi_{(E2)}$) of Pos- and PEGylated-laccase of the enzyme mixture. ○: measured with error bars (SD), (----): predicted by Eq. (15), (—): predicted by Eq. (17), and (····): predicted by Eq. (16).

$$M=M_1\varphi_1+M_2\varphi_2=M_1(1-\varphi_2)+M_2\varphi_2 \quad (12)$$

$$M^{-1}=M_1^{-1}\varphi_1+M_2^{-1}\varphi_2=M_1^{-1}(1-\varphi_2)+M_2^{-1}\varphi_2 \quad (13)$$

$$\log M=\varphi_1 \log M_1+\varphi_2 \log M_2=(1-\varphi_2) \log M_1+\varphi_2 \log M_2 \quad (14)$$

Table 2 B_f and A_f values of the statistical indices of three mixed rules for the estimation of Arrhenius activation energy (*E*a) according to the ratio of enzyme mixture

Equation	B_f[a]	A_f[b]
$Ea_{mix}=Ea_{E1}(1-\varphi_{(E2)})+Ea_{E2}\,\varphi_{(E2)}$	0.9956	1.0115
$Ea_{mix}{}^{-1}=Ea_{E1}{}^{-1}(1-\varphi_{(E2)})+Ea_{E2}{}^{-1}\varphi_{(E2)}$	1.0443	1.0443
$\log Ea_{mix}=(1-\varphi_{(E2)})\log Ea_{E1}+\varphi_{(E2)}\log Ea_{E2}$	1.0196	1.0196

[a]Bias factor.
[b]Accuracy factor.

where M is the modulus and φ is the volume ratio. Eqs. (12) and (13) are the upper bound and lower bound models, respectively. Eq. (14) is the logarithmic mixture model. We can substitute Ea_{mix} for M, and Ea_{E1}, Ea_{E2}, for M_1, M_2, respectively. Furthermore, φ_1 and φ_2 were regarded as $\varphi_{(E1)}$ and $\varphi_{(E2)}$, a fraction of the enzymes 1 (*E1*) and 2 (*E2*), respectively. Thereafter, Ea_{mix} to $\varphi_{(E2)}$ (the ratio of enzyme mixture) was calculated and compared with their corresponding measured values as follows:

$$Ea_{mix}=Ea_{E1}\,\varphi_{(E1)}+Ea_{E2}\,\varphi_{(E2)}=Ea_{E1}(1-\varphi_{(E2)})+Ea_{E2}\,\varphi_{(E2)} \quad (15)$$

$$Ea_{mix}{}^{-1}=Ea_{E1}{}^{-1}\varphi_{(E1)}+Ea_{E2}{}^{-1}\varphi_{(E2)}=Ea_{E1}{}^{-1}(1-\varphi_{(E2)})+Ea_{E2}{}^{-1}\varphi_{(E2)} \quad (16)$$

$$\log Ea_{mix}=\varphi_{(E1)}\log Ea_{E1}+\varphi_{(E2)}\log Ea_{E2}=(1-\varphi_{(E2)})\log Ea_{E1}+\varphi_{(E2)}\log Ea_{E2} \quad (17)$$

Using the equations described above, the exact ratio of the enzyme mixture can be calculated to design the best TTI formulation. Therefore, three-mixture rule must be applied to evaluate their performance. Table 2 shows the B_f and A_f values of the statistical indices for three-mixture rules of *E*a vs. $\varphi_{(E2)}$. All B_f values obtained were in the range of 0.9956–1.0443, which were close to unity, indicating a perfect concordance. Therefore, the B_f values obtained in our study were acceptable and indicated that there was only a minimal difference between the predicted and observed data. For the accuracy factor (A_f=1.00, if a model fits perfectly), the simple mixture rule showed the closest to unity. Considering B_f and A_f together, the simple mixture rule ($Ea_{mix}=Ea_{E1}\,\varphi_{(E1)}+Ea_{E2}\,\varphi_{(E2)}$) appeared to fit the data best. Using the equation described above, the exact ratio of the enzyme mixture can be calculated to design the best TTI formulation.

Evaluation of the applicability of a mixed laccase prototype TTI under dynamic storage condition. Food products experience various time-temperature changes during storage and distribution under roughly controlled refrigeration condition. Therefore it is important to examine how well the customized TTI predicts the actual quality changes of a food quality factor under temperature abuse condition. Using Eq. (15), *E*a of a typical mixed laccase TTI ($\varphi_{(E2)}$=0.5) was calculated to be 38.71 kJ/mol. Prototype TTIs prepared as described in Materials and Methods were stored in a low-temperature programmable incubator (5–15°C) interfaced to automatic temperature controller, and absorbance of the TTI were measured at fixed time intervals. Fig. 4 shows the predicted and measured values. Predicted simulation curve was derived using Arrhenius equation substituting *E*a and *A* for 38.71 kJ/mol and 1.7×10^6 s^{-1} as follows: $\nu=Ae^{Ea/RT}$, where ν is a rate of reaction (s^{-1}), A is the pre-exponential factor (s^{-1}), *E*a is the activation energy (kJ/mol), R is the ideal gas constant, and T is the absolute temperature (K). The simulated curve and measured values increased consistently and similarly up to 40 h, during which relative errors of the predicted and observed values were within ±5% error bar. Small deviation between observed and predicted values was found after about 50 h. This could be due to the denaturation of Pos-laccase enzyme during storage, and the apparent rate of reaction catalyzed by the enzyme mixture would tend to decrease gradually during storage. Consequently, the customized TTI formulation appeared to predict the change of temperature history and the total accumulation of exposure to heat up to 40 h stored at 5–15°C with an acceptable accuracy. Further study should be made to enhance the stability of the native enzyme to increase the effective range of reliable prediction.

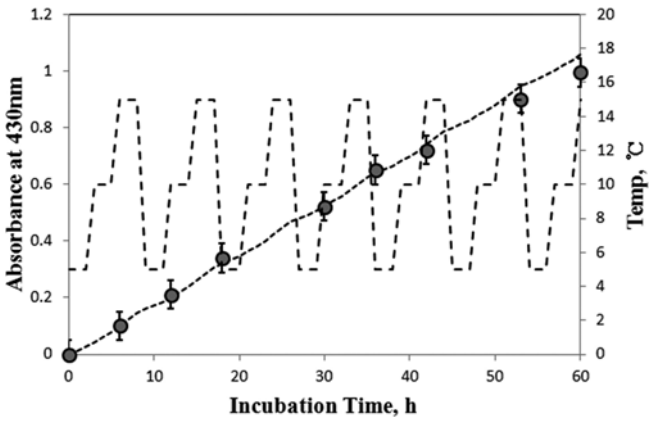

Fig. 4 Comparison of time course of predicted and measured absorbances (abs) of the enzyme mixture ($\varphi_{(E2)}$=0.5) under dynamic storage condition (5–15°C). ●: measured abs with error bars (SD), (…): predicted abs, (---): temperature inside the low-temperature incubator.

In conclusion, relationship between *E*a and ratio of the enzyme mixture was not only linear, but also it was well fitted to the linear mixture rule. This newly found linear dependency is different from kinetically derived one in the case of the laccase TTI. Therefore, it will be necessary to carry out similar experiments for other isozymes to confirm whether mixture rules hold true or not, and if so, which mixture rule is fitted best to the experimental data. This infers that prior to using certain enzyme mixture systems, a properly designed experiment should be performed to ensure which mixture rule is best applicable. The present study will open the way for practical and economical application of the enzyme mixture system by applying the simple mixture rule on the adjustment of *E*a for enzymatic TTIs.

Acknowledgments This research was supported by R&D Convergence Center Support Program (710003-03) of the Ministry for Food, Agriculture, Forestry and Fisheries, Korea. The authors wish to thank the 3M Korea for the supply of material and financial support.

References

Bobelyn E, Hertog ML, and Nicolaï BM (2006) Applicability of an enzymatic time temperature integrator as a quality indicator for mushrooms in the distribution chain. *Postharvest Biol Tec* **42**, 104–14.

Casal JA, Chabas A, and Tutor JC (2003a) Thermodynamic determination of β-hexosaminidase isoenzymes in mononuclear and polymorphonuclear leukocyte populations. *Am J Med Genet* **116A**, 229–33.

Casal JA, Perez LF, and Tutor JC (2003b) Thermodynamic determination of plasma and leukocyte beta-hexosaminidase isoenzymes in homozygote and heterozygote carriers for the GM_2 gangliosidosis B1 variant. *Am J FOR Clin Pathol* **119**, 684–8.

Dalgaard P and Jorgensen LV (1998) Predicted and observed growth of *Listeria monocytogenes* in seafood challenge tests and in naturally contaminated cold smoked salmon. *Int J Food Microbiol* **40**, 105–15.

Giannakourou MC, Koutsoumanis K, Nychasc GJE, and Taoukis PS (2001) Development and assessment of an intelligent shelf life decision system for quality optimization of the food chill cain. *J Food Protect* **64**, 1051–7.

Giannakourou MC and Taoukis PS (2003) Application of a TTI-based distribution management system for quality optimization of frozen vegetable at the consumer end. *J Food Sci* **68,** 201–9.

Giannakourou MC, Koutsoumanis K, Nychasc GJE, and Taoukis PS (2005) Field evaluation of the application of time temperature integrators for monitoring fish quality in the chill chain. *Int J Food Microbiol* **102**, 323–36.

Guiavarc'h Y, Sila D, Duvetter T, Loey AV, and Hendrickx M (2003) Influence of sugars and polyols on the thermal stability of purified tomato and cucumber pectinmethy lesterases: a basis for TTI development. *Enzyme Microb Technology* **33**, 544–55.

Huber P and Carré B (2012) Decolorization of process waters in deinking mills and similar applications: A review. *BioResources*, **7**, 1366–82.

Kim K and Lee SJ (2009) Effects of extraction on κ-carrageenan/agar mixed gel type. *J Texture Stud* **40**, 646–56.

Kim K, Kim EJ, and Lee SJ (2012a) New enzymatic time-temperature integrator (TTI) that uses laccase. *J Food Eng* 113, 118–23.

Kim W, Park EN, and Hong KW (2012b) Development of a time-temperature integrator system using *Burkholderia cepacia* lipase. *J Food Sci Biotechnol* **21**, 497–502.

Kunamneni A, Ghazi I, Camarero S, Ballestereos A, Plou FJ, and Alcalde M (2008) Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. *Process Biochem* **43**, 169–178.

López-Cruz JI, Viniegra-González G, and Hernández-Arana A (2006) Thermostability of native and pegylated *Myceliophthora thermophila* laccase in aqueous and mixed solvents. *Bioconjugate Chem* **17**, 1093–8.

Park HR, Kim K, and Lee SJ (2013) Adjustment of Arrhenius activation energy of laccase-based time–temperature integrators (TTIs) using sodium azide. *Food Control* **32**, 615–20.

Ross T (1996) Indices for performance evaluation of predictive models in food microbiology. *J Appl Bacteriol* **81**, 501–8.

Taoukis PS and Labuza TP (1989) Reliability of time-temperature indicators as food quality monitors under nonisothermal Conditions. *J Food Sci* **54,** 789–92.

Taoukis PS, Koutsoumanis K, and Nychas GJE (1999) Use of time–temperature integrators and predictive modeling for shelf life control of chilled fish under dynamic storage conditions. *Int J Food Microbiol* **53,** 21–31.

Taoukis PS (2001) In *Food Process Modelling*: *Modelling the Use of Time–Temperature Indicators in distribution and stock rotation*, CRC Press, Washington DC.

Tsoka S, Taoukis PS, Christakopoulos P, Kekos D, and Macris BJ (1998) Time temperatüre integration for chilled food shelf life monitoring using enzyme-substrate systems. *Food biotechnol* **12**, 139-155.

Vaikousi H, Biliaderis CG, and Koutsoumanis KP (2009) Applicability of a microbial time temperature indicator (TTI) for monitoring spoilage of modified atmosphere packed minced meat. *Int J Food Microbiol* **133**, 272–8.

Yan S, Huawei C, Limin Z, Fazheng R, Luda Z, and Hengtao Z (2008) Development and characterization of a new amylase type time–temperature indicator. *Food Control* **19**, 315–9.

Zakhartsev M, Johansen T, Pörtner HO, and Ronny B (2004) Effects of temperature acclimation on lactate dehydrogenase of cod (*Gadus morhua*): genetic, kinetic and thermodynamic aspects. *J Exp Biol* **207**, 95–112.

Zhao X, Jiang H, Wang H, Zhao J, Qiu Q, Tapper N et al. (2013) Remotely sensed thermal pollution and its relationship with energy consumption and industry in a rapidly urbanizing Chinese city. *Energy Policy* **57**, 398–406