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A modified titrimetric method for measuring the activity of wheat germ lipase

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RESEARCH ARTICLE

Abstract

Wheat germ lipase is a cereal lipase which plays an important role in the growth and storage of wheat products. The commonly used titrimetry is not reliable and accurate enough to determine lipase activity due to its difficulties in identifying the end point when used to measure the alkaline lipase of low activity. In this paper, acidic titration method was utilised, with triacetin as substrate to measure the activity of wheat germ lipase by an automatic potentiometric titrator. The optimum catalysis time is 4 h, the pH value of end point is 6.0, and the initial pH of substrate is 7.7. Under optimum test conditions, the activity of wheat germ lipase was found to be 50.398±0.848 U/mg, within a linear range of 1.0-10.0 mg, and with relative standard deviation equal to 1.682%. This method has proved to be precise and reproducible for measuring alkaline lipase of low activity in aqueous solution.

Keywords: wheat germ lipase, triacetin, titration, method

1. Introduction

Lipase activity (LA) is a common evaluation criterion that can be used to access the safety and quality of grain (Xu *et al.*, 2013). The accuracy of LA determination is closely related to various factors, including methods, conditions, and substrates.

There are different methods for determining LA, which include: (1) titrimetry; (2) colorimetric assays; (3) chromatographic procedures (thin layer chromatography, gas chromatography, high performance liquid chromatography); (4) nephelometry and turbidimetry; and (5) electric conductivity. Among these approaches, the titrimetric method is relatively feasible and cost effective (Aravindan *et al.*, 2007; Gupta *et al.*, 2003), and has therefore become the most extensively used technology for LA determination (Gupta *et al.*, 2003; Hasan *et al.*, 2009).

Olive oil, triolein, and tributyrin are commonly used substrates for the determination of LA using titration (Ebonogue *et al.*, 2006; Gilham and Lehner, 2005; Mateos *et al.*, 2007). As previously reported, lipase catalytic ability was measured by using emulsified olive oil as the substrate (Kapranchikov *et al.*, 2004; Korneeva *et al.*, 2008). Olive oil has been certified as a standard substrate used in LA assays in European Pharmacopeia and gum arabic is usually used for emulsification (Stoytcheva *et al.*, 2012). However, the preparation procedures of olive oil emulsions for LA assay are complicated (ultrasonic wave equipment needed), the emulsions are not stable, and the results are not consistently reproducible, making this method defective. To overcome the drawbacks of LA determined using olive oil, short chain glycerides, such as triacetin (Akoh *et al.*, 2004) and tributyrin (Rizzello *et al.*, 2010), are more popular substrates in such research. Short chain glycerides can be dispersed sufficiently in buffer solution to form a stable oil-water interface without the aid of emulsifying agent; in addition, the substrate solution is clear and transparent. These advantages make short chain glycerides a suitable substrate for titrimetric analysis.

Moreover, in conventional LA assays, researchers usually neutralise free fatty acids (FFAs) with NaOH to maintain pH at a constant alkaline end point value (Rajeshwara and Prakash, 1996; Wang *et al.*, 2012; Zarrintan *et al.*, 1990) because long chain FFAs such as oleic acids have a high pKa, and the reaction can only be fully completed during

titration when pH>9. On the contrary, short chain FFAs such as butyric acid can be completely titrated when the pH value is about 6 (Stoytcheva *et al.*, 2012), and short chain glycerides are susceptible to hydrolysis under alkaline conditions. Accordingly, it is difficult to observe the end point by alkalimetry that uses short chain glyceride as a substrate. The above facts show that there is a need for a method of optimisation. Wheat germ lipase has good thermal stability and remains active in organic solvents, and is thus widely utilised in the pharmaceutical, chemical, and food industries (Ahmad *et al.*, 2010; Pierozan *et al.*, 2011). Wheat germ lipase is primarily composed of a monomeric protein (about 42 kDa) (Fadlloglu and Soylemez, 1996) that is maximally active at 37 °C (Kapranchikov *et al.*, 2004) and pH=7.7. When pH decreases to 7.0, LA is lowered by 50%. A high pH value (>10) causes unwinding of the α-helix, leading to a loss of enzyme activity (Sudhindra *et al.*, 1991). All these characteristics of wheat germ lipase lay the theoretical foundation for titrimetric methods in the determination of LA. In this research, the activity of wheat germ lipase was studied using triacetin (substrate) and automatic potentiometric-titrator. On the basis of determination, the conventional titrimetric method was optimised, by which a simple and effective method for measuring LA is established with short chain glyceride as the substrate.

2. Materials and methods

Reagents

Wheat germ lipase (5-15 U/mg, type I, cat. no. L3001) and triacetin were purchased from Sigma-Aldrich (Steinheim, Germany). Other reagents used in the paper were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China P.R.).

Preparation of enzyme solution and substrate

Wheat germ lipase was dissolved in 0.1 mol/l Tris-HCl buffer (pH=7.7) to prepare 2 mg/ml enzyme solution for assays. Triacetin was added to 0.1 mol/l Tris-HCl buffer to prepare 0.3 mol/l substrate solutions.

Stability analysis of triacetin in buffers with different pH

Triacetin was added to 0.1 mol/l Tris-HCl buffers (pH=3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) to prepare 0.3 mol/l substrate solutions. After which the buffers were incubated in a hot air oven at 37 °C. The pH values were measured every 72 h.

Method for measuring lipase activity

Substrate was transferred into the sample and blank tubes (10 ml/tube), respectively. Lipase solution (2.0 ml) was added to the sample tube, while the same volume of buffer was added to the blank tube. After incubation at 37 °C, enzyme catalytic reactions in tubes were prevented immediately. Then the mixtures in the sample and blank tubes were titrated against 0.005 mol/l H_2SO_4 until the same end point was achieved (pH is given in 'Optimisation of assay conditions for LA'). According to the difference between the volumes of titrant used (ΔV_{H2SO4}), the amount of FFAs in the tubes released from triacetin by lipase catalysed can be calculated. H_2SO_4 titration was conducted on a Mettler Toledo S220 automatic potentiometric titrator (Mettler Toledo Inc., Columbus, OH, USA) to ensure the accuracy of the titration process.

One unit of LA was defined as the amount of FFA liberated from 1.0 mg triacetin per hour, pH=7.7, T = 37 °C (the optimum pH for wheat germ lipase is 7.7; Kapranchikov *et al.*, 2004). The formula of LA is as follows:

$$LA = \frac{B - A}{t \times m \times n} \quad (1)$$

Where LA = lipase activity (U/mg); A = sample consumption volume of standard H_2SO_4 solution for titration (ml); B = blank sample consumption volume of standard H_2SO_4 solution for titration (ml); t = reaction time (h); m = the amount of H^+ in standard 0.005 mol/l H_2SO_4 solution (μmol); and n = dilution multiples of lipase solution.

Optimisation of assay conditions for lipase activity

Selection of end point

The terminal point pH of titration was set at 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0, respectively. 2.0 ml lipase solution was added to sample tubes, while 2.0 ml Tris-HCl buffer was added to blank tubes. They were incubated in an oven at 37 °C for 4 h, and cooled down in ice water for 3 min to stop enzyme-catalysed reactions. They were then titrated with 0.005 mol/l H_2SO_4. The concentration of Tris-HCl buffer was 0.1 mol/l and the initial pH was 7.7.

Effect of reaction time on lipase activity

The catalysis time was set at 1, 2, 4, 8, 10, and 12 h, respectively, and the pH of the titration end point was selected at pH=4.5 (the reason for this was that the pH of the reaction system would drop as the reaction time increased, therefore, a pH lower than the reaction system was needed), while the other procedures were the same as above ('Selection of end point'), and 0.005 mol/l H_2SO_4 was used to measure LA.

Effect of initial pH of substrate on lipase activity

The initial pH of substrate (Tris-HCl buffer) was set at 7.0, 7.5, 8.0, 8.5, 9.0, respectively, and the terminal point pH of titration was 6.0, while other procedures were the same as above ('Selection of end point'), and 0.005 mol/l H_2SO_4 was used to measure LA.

Effect of buffer concentration on lipase activity

The concentration of Tris-HCl buffer solution was set at 0.05, 0.10, 0.15, 0.20, and 0.30 mol/l, respectively, and the terminal point pH of titration was 6.0, while other procedures used in determination were the same as above mentioned methods ('Selection of end point'), and 0.005 mol/l H_2SO_4 was used to measure LA.

Effect of termination method on lipase activity assayed

After all the tubes were incubated in an oven at 37 °C for 4 h, three kinds of reagents were tested to prevent the reaction. They were ethanol, propanol, and isopropanol. When the reaction was over, 10 ml reagent was added to partial tubes; other sample tubes were heated in 90 °C water for 10 min, or cooled down in ice water for 3 min to stop enzyme-catalysed reactions. Other procedures were the same as above ('Selection of end point'), and 0.005 mol/l H_2SO_4 was used to measure LA.

3. Results and discussion

Stability of triacetin in Tris-HCl buffer solution

The stability of triacetin in Tris-HCl buffer of different pH values is shown in Figure 1. As can be seen, when the initial pH is 9.0 or 10.0, the substrate hydrolyses fast, while the rate of triacetin hydrolysis is nonetheless low when the initial pH is below 7.0. Generally, the non-enzymatic hydrolysis in buffer solution occurs at a pH higher than 5.0. The results showed that the rates of non-enzymatic hydrolysis of triacetin differentiated with initial pH values. Under acidic and neutral conditions, triacetin is more stable.

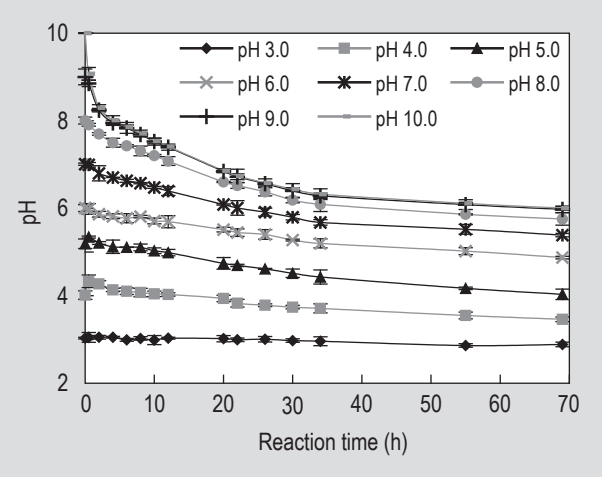

Figure 1. Stability of triacetin in Tris-HCl buffer of different pH value. Data are reported as mean value ± standard deviation (n=4).

The reason why substrate is unstable under alkaline conditions is that triacetin is a kind of ester which can react with alkali to form alcohols leading to a continuous decrease in pH value. The pH of substrate solution will keep dropping unless the substrate is completely consumed by reacting with alkali in a short time. However, it is known that the excessive volume of substrate is required when the enzyme activity is assayed. This explains why the end point of pH is hard to reach when the FFAs are titrated by alkali. It also explains why the LA value from the conventional titrimetric method is unstable. On the contrary, the substrate stays stable in an acidic system; acidimetry for the determination of LA may reduce the measurement error caused by non-enzymatic hydrolysis of substrate. Consequently, the acidimetry is preferable when the substrate is triacetin. The optimisation of this acidimetry for determining LA will be described in the following sections.

Selection of end point for titration

This study aims to establish an acidic titration method for measuring LA. The pH of the reaction system itself should be considered before setting the end point. Due to different titration end points, the amount of H_2SO_4 needed to titrate sample tubes and blank tubes varies. Non-enzymatic hydrolysis and lipase catalysed hydrolysis of substrate will lead the pH of the reaction system to drop rapidly as the incubation continues. When H_2SO_4 was used to titrate samples, a lower pH of end point had to be chosen.

Figure 2A shows the relationship between the amount of H_2SO_4 for titration and the end point pH. As shown, the amount of H_2SO_4 used significantly increases with a decrease in end point for titration pH. And when the titration end point is 7.0, the volume of H_2SO_4 used to titrate blank tube is almost zero which will introduce a large error in the determination results. Therefore the titration end point must be lower than 7.0. Additionally, when the end point of titration is below 5.0, the amount of H_2SO_4 needed for sample and blank tubes is greater than 10 ml; when the pH of end point is 3.0, the volume of H_2SO_4 used is over 20 ml, and ΔV_{H2SO4} is negative. From Figure 2B, it could be noted that when the pH is 6.0-7.0, a low volume of H_2SO_4 is used for titration and the LA value determined is stable. There is no significant difference in the amount of ΔV_{H2SO4} consumed between pH=6.0 and 7.0. However, LA value decreases with a decrease in the pH of titration end point.

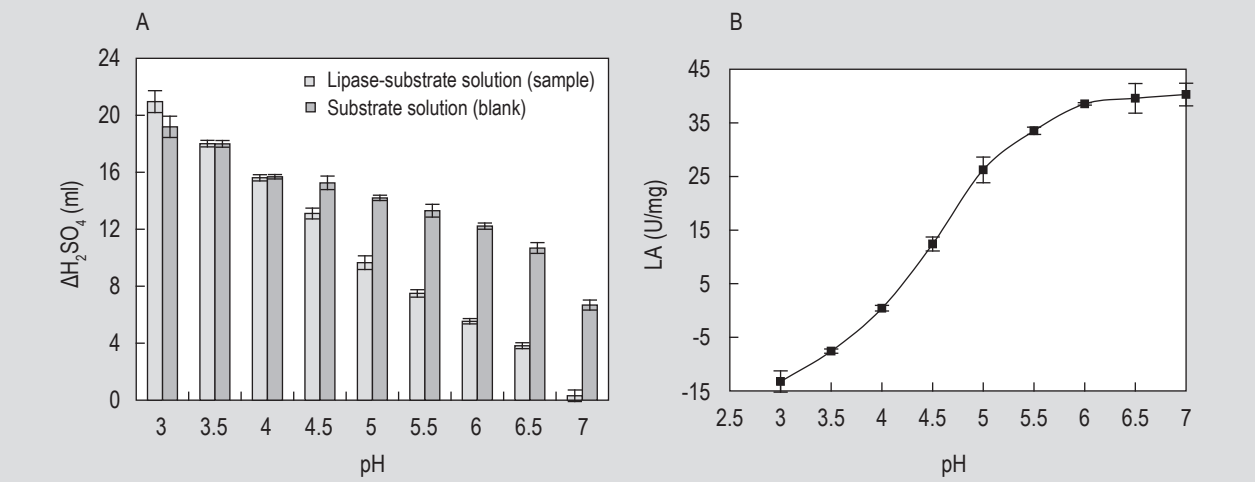

Figure 2. (A) Relationship between the amount of H_2SO_4 for titration and the end point pH. (B) Relationship between end point pH and the results of lipase activity (LA) assay. Data are reported as mean value ± standard deviation (n=3).

Gupta *et al.* (2003) has studied the above phenomenon. His studies showed that, under low pH conditions, free fatty acids were in a state of incomplete dissociation, which led to the decrease in ΔV_{H2SO4} consumed. But that did not explain the negative ΔV_{H2SO4}. In a blank tube, the drop in pH can only be attributed to the self-hydrolysis of substrate. Comparatively, it is wheat germ lipase catalysed hydrolysis and non-enzymatic hydrolysis that contribute to a lower pH in the sample tube. Since the initial pH of the sample tube should be more acidic than the blank tube, the H_2SO_4 used to titrate it should be less than that used to titrate the blank tube. This abnormal result may be due to the fact that the sample tube requires more titrant at pH=3-4 because of the pKa of acetic acid; the wheat germ lipase releases more acetic acid which acts as a buffer at these pH values and this requires more titrant to reach the desired pH than in the blank tube with less acetic acid. Conclusively, the pH of end point of acidic titration should not be set too low for the determination of LA.

In a word, the assay value of LA is closely related to the titration end point. And the results showed that pH=6.0 is the optimum end point for the titration.

Effect of reaction time on lipase activity assay

Wheat germ lipase catalyses the hydrolysis of triacetin to release FFAs. Figure 3A shows the relationship between the reaction time and the pH value of the reaction system. The pH in both of the sample and blank tubes decreased with an increase in reaction time (Figure 3A). This is probably due to the catalysis of lipase, and the decrement in pH of sample after 12 h of the catalysis of lipase. But, the pH in the sample and blank tubes does not change. LA decreases after reaction for 12 h as the substrate is massively consumed. The non-enzymatic hydrolysis of substrate is responsible for the drop in pH. Thus, the reaction time should be controlled in 12 h for determining LA.

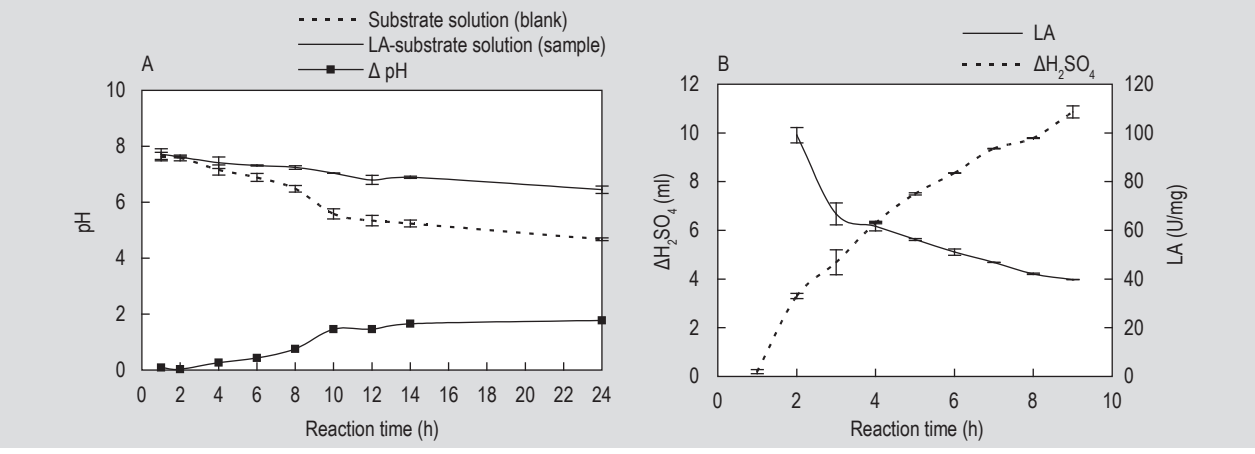

Figure 3. (A) Relationship between the reaction time and the pH value of the reaction system. (B) Relationship between lipase activity (LA) (usage of H_2SO_4 titrant) and reaction time. Data are reported as mean value ± standard deviation (n=4).

From Figure 3A, it could also be noted that the longer the reaction lasts, the higher is the ΔV_{H2SO4}. After calculation, Figure 3B indicates LA drop as reaction continues. There are two main reasons for this phenomenon. Firstly, enzyme activity is closely related to the concentration of the substrate, and thus the drop in the LA is mainly due to limited amounts of substrate. Secondly, enzyme activity is closely related to its optimum pH for catalysis. The FFAs released from hydrolysis of triacetin inhibit LA in return. Moreover, if the reaction time is too short, the ΔV_{H2SO4} will be less and thus the measurement error will be subsequently increased. The pH of the reaction system declined steadily, and the pH is higher than the end pH=6.0 with 4-6 h (Figure 3A and 3B). So, 4-6 h is suitable as a titration end point. To save time, 4 h is ultimately selected for the optimum reaction time of LA determination.

Figure 3A also reveals that the amount of H_2SO_4 titrant used in both sample and blank tubes constantly increases as the reaction extends beyond 12 h. This will cause a significant error when a large amount of samples is assayed. Therefore, it is advisable to prevent the enzymatic reaction by terminator or other means before assaying the product.

Effect of initial pH of substrate on lipase activity assay

The optimum pH for wheat germ lipase is 7.7. Thus, pH values between 7.0 and 9.0 are defined as suitable (Kapranchikov *et al.*, 2004). On this basis, the pH of Tris-HCl buffer, used for preparing the substrate solution, is set to be 7.0, 7.5, 8.0, 8.5, and 9.0, and the pH of end point is 6.0. The differences among these groups are determined according to the results from LA assays after a reaction rate of 4 h.

Figure 4 shows that assay results of LA decrease with a rise in initial pH of substrate. And there is a significant difference (P<0.05) between the first and last group. The initial pH of the substrate in group 1 is 7.0. When the reaction is completed, the final pH is close to the end point, which easily causes errors. When the initial pH is 7.5, 8.0, or 8.5, the results have no significant difference (P>0.05), while when the initial pH is 9.0, the LA assayed decreases significantly. This implies that a high pH inhibits LA within this pH range. Titration error can be reduced when the initial pH of substrate is far away from the end point of titration. Therefore, pH=7.0 is not a good choice as it is close to the end point. Given the above observations, it is concluded that the best initial pH of substrate is 7.7 for the enzyme.

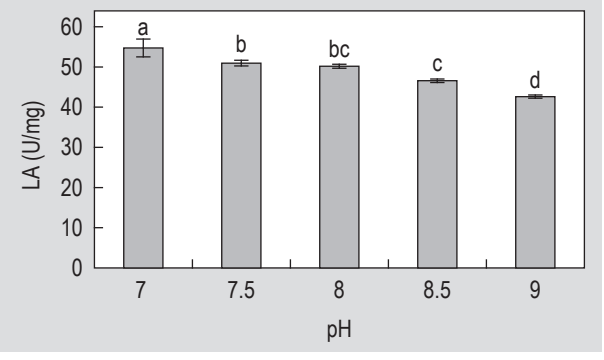

Figure 4. Relationship between initial pH of substrates and the results of lipase activity (LA) assay. Letters above the columns represent significant difference between groups (P<0.05). Data are reported as mean value ± standard deviation (n=3).

Effect of buffer concentration on lipase activity assay

The concentration of buffer solution can affect the pH of the reaction system during the catalytic process. At the same time, the change in rate of pH most likely affects the results of the assay. As shown in Figure 5A, assay results of LA increase with an increase in buffer concentration. The change in Tris-HCl buffer concentration caused a significant difference between the first and last group (P<0.05). When the concentrations are 0.1, 0.15, or 0.2 mol/l the results have no significant difference (P>0.05).

Figure 5B shows relationship between the concentration of Tris-HCl buffer and the amount of H_2SO_4 for titration. As shown, the amount of H_2SO_4 used is comparatively less (using 0.05 mol/l concentration) than buffer, which easily leads to error in results. In buffer with higher concentration, the LA assayed is relatively higher. However, it not only requires more H_2SO_4 to titrate the products in the sample and blank tubes, but also extends the titration time. Thus, 0.1 mol/l Tris-HCl is considered as the best buffer solution for LA determination.

Effect of termination method on lipase activity assay

Organic reagents cause enzyme denaturation by destroying the hydration layer of protein in aqueous solution. In this way, they stop enzyme catalysed reactions (Khmelnitsky *et al.*, 1991). Ethanol, propanol and isopropanol are common terminators of catalytic reactions (Kermasha *et al.*, 1993; Prazeres *et al.*, 2006). Water bath (conventional heating system) can cause loss of enzyme activity, while ice water can reduce enzyme activity. In this part, the effects of the above termination methods on LA value are tested. Figure 6A shows that LA assayed decreases after the treatment of organic reagents and conventional heating. The lipase sample was tested to be more catalytically active in the group with ice water treatment. The use of different termination methods leads to a lower assayed value of the LA. As shown in Figure 6B, more H_2SO_4 is used for titration after the addition of organic reagents. In comparison, less H_2SO_4 is needed when the sample is heated in a water bath.

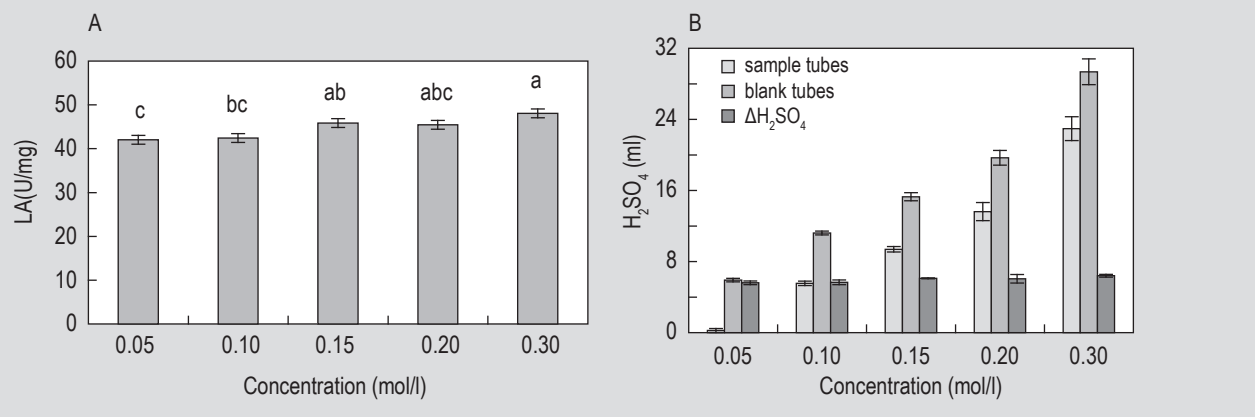

Figure 5. (A) Relationship between the concentration of Tris-HCl buffer and assay results of lipase activity (LA). (B) Relationship between the concentration of Tris-HCl buffer and the amount of H_2SO_4 for titration. Data are reported as mean value ± standard deviation (n=3).

The reason why samples adding organic solvents as terminators need an obviously larger amount of H_2SO_4 to titrate is probably that the organic solvents can destroy the balance of hydrogen ions in the aqueous solution, causing a negative deviation of lipase titration results (Figure 6). Compared with water, organic solvents such as ethanol, acetone and isopropyl alcohol have weaker affinity for protons. As a result, in order to reach the same pH, more H_2SO_4 needs to be used. In addition, Coulomb's law indicates that the attraction of opposite charges is inversely proportional to the dielectric constant of solvents. Acids produced by the substrate will dissociate and give out a proton to solvent molecules, and ion pairs are formed by the two molecules through electrostatic attraction. Dielectric constants of three organic reagents in this research were lower than that of water (water > ethanol > isopropyl alcohol > acetone). When they were added to the reaction product, the attraction of opposite charges increased, leading to the formation of ion pairs. Therefore, the proton dissociation of acetic acid, the reaction product, is weakened. As the acidity of solution decreased, more H_2SO_4 is needed to achieve the end pH of titration. Conventional heating also achieves titration results because of the volatilisation of acetic acid during the process. All the methods mentioned above can stop the catalysis and the choice should be made depending on the amount of sample.

However, it is reported that the assayed results are stable and reproducible with the use of organic reagent. Researchers commonly used one reagent in the process of determining the LA to stop enzyme catalysed reactions (Jiang *et al.*, 2007; Kermasha *et al.*, 1993). In our further study, the stabilities of different terminators on LA assay results were compared and analysed by titrating the samples immediately and 2 h later. As shown in Figure 6A, with the aid of organic reagent or conventional heating, the assayed results are more stable than ice water. According to Figure 6B, H_2SO_4 used for titration remains almost the same by titrating the samples immediately and 2 hours later, which indicates that organic chemicals can inactivate lipase effectively. When massive samples are to be assayed, the use of the above termination method is preferable to ice water. On the other hand, ice water treatment is suitable when the number of samples is small.

Linear range and precision

Different amounts of lipase solution samples are measured between 0.5-5.0 ml, which is equivalent to 1.0-10.0 mg lipase. The amount of added lipase solution and titrant used is well linearly related: $y = 3.1755x - 0.2029$, $R^2=0.9991$ (x = the amount of added lipase solution; y = the volume of H_2SO_4 titrant). Assay of LA was repeated 6 times under the optimum conditions. LA is calculated to be 50.398±0.848 U/mg, standard deviation = 1.682% (data not shown).

4. Conclusions

The optimised condition for determining LA is when 2.0 ml lipase solution is incorporated with 10.0 ml triacetin solution that is dissolved in Tris-HCl buffer (buffer pH is 7.7 and concentration is 0.10 mol/l, substrate solution is 0.30 mol/l). The catalytic reaction is maintained at 37 °C for 4 h. The product is titrated with 0.005 mol/l H_2SO_4 until the end point (pH=6.0). LA is converted by the usage of H_2SO_4.

Under this condition, LA assayed is 50.398±0.848 U/mg, standard deviation = 1.682%. Also, when the quantity of added lipase is in the range of 1.0-10.0 mg, the linearity of titrimetric analysis is good.

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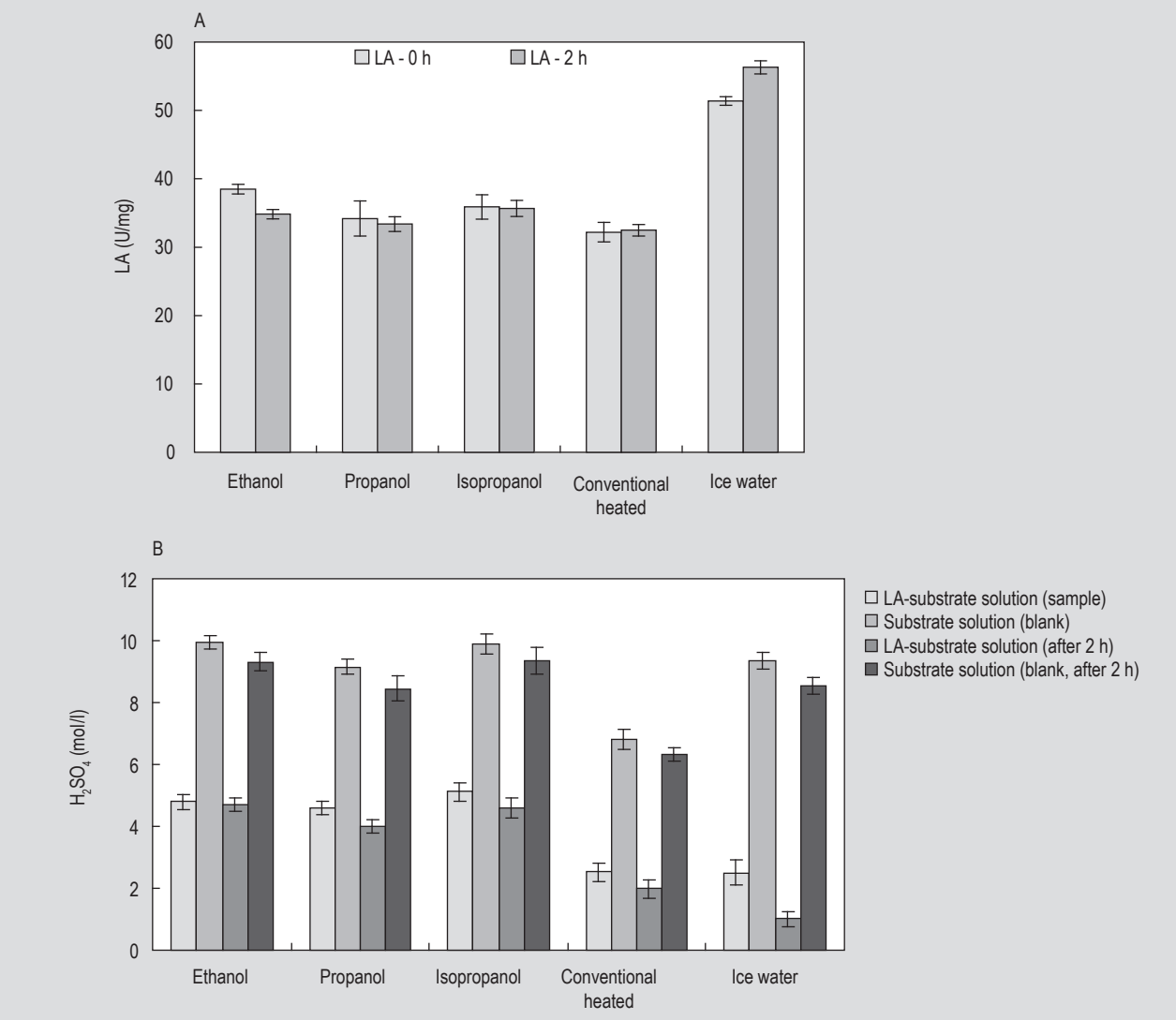

Figure 6. (A) Comparison of the stabilities of assayed lipase activity (LA) after different terminate methods (LA either determined immediately after adding terminators or 2 h after adding terminators). (B) Relationship between the terminate methods and the usage of H_2SO_4 for titration. Data are reported as mean value ± standard deviation (n=3).

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