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A modified colorimetric method for determining the activity of wheat germ lipase in low-aqueous media

B. Xu[1], W.-J. Miao[1], Q.-S. Hu[2], C. Gao[3] and Y. Dong[1]

[1]School of Food and Biological Engineering, Jiangsu University, Zhenjiang, 212013, China P.R.; [2]School of Materials Science and Technology, Nanjing University of Aeronautics and Astronautics, Nanjing, 210015, China P.R.; [3]School of Life science and Technology, China Pharmaceutical University, Nanjing, 210009, China P.R.; xubin@ujs.edu.cn

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RESEARCH PAPER

Abstract

Copper soap colorimetry is a common way to determine the free fatty acids of grain. However, when determining the lipase activity of grain by copper soap colorimetry its accuracy is frequently affected by the degree of mixedness between lipase and substrate. Grain lipase extraction and purification steps are quite cumbersome as well. To overcome these shortcomings, a modified colorimetric method was established for the determining of wheat germ lipase activity in a low-aqueous media. First, we partially defatted wheat germ, then adjusted it to a certain water activity, added some olive oil, extracted the free fatty acids after incubating for a period of time, then determined the absorbance of reaction system by copper soap colorimetry. Finally we obtained the activity of wheat germ lipase by calculation. The optimum conditions were: coordinated the water activity of wheat germ to 0.84, the addition of 0.75 ml olive oil per gram wheat germ and incubation for 4 h at 55 °C. It was calculated that the lipase activity was 3.25 U/g, and the relative standard deviation was 5.82% (n=10). This method eliminates the steps of lipase extraction and purification. Compared with the traditional methods, it is simple, rapid and reproducible.

Keywords: activity, copper soap colorimetry, lipase, low-aqueous media, water activity, wheat germ

1. Introduction

Wheat germ is a kind of by-product of wheat processing. It contains various nutrient substance and active ingredients such as lipase (LA) and lipoxyenase (LOX) which readily lead to rancidity. As a result, wheat germ is most commonly used as feed. If we want to make full use of this valuable resource, it is essential to inactivate the endogenous enzymes. However, the study of wheat germ stabilization is based on the accurate determination of LA and LOX activity. Since a study on ultraviolet rapid determination of wheat germ LOX activity has been reported (Xu *et al.*, 2012), this report lays emphasis on the rapid determination of LA activity.

Generally, there are 3 steps in grain LA activity determination:

- Extract LA from grain;
- Mix LA extraction with substrate properly to incubate free fatty acids (FFA);
- Determine the activity of LA from the amount of FFA produced from LA hydrolysis reaction.

There have been series of FFA determination methods developed so far, among which titration method (Lam and Proctor, 2001; Ramezanzadeh *et al.*, 1999), colorimetric method (Goffman and Bergman, 2003) and chromatography (Nishiba *et al.*, 2000). Among them, the colorimetric method, especially copper soap colorimetry for determination of the FFA content in grain, has the advantages of rapidness, high sensitivity and lower apparatus cost and for these reasons it is favoured by analysts. The method has been improved several times. Baker obtained copper soap through the reaction between 5% copper acetate and FFA in grain and then determined the content of FFA by absorbance (Baker, 1961). Duncombe (1963) took $Cu(NO_3)3H_2O$ as a copper reagent and triethanolamine as a chromogenic reagent.

Lowry and Tinsley (1976) used acetic copper-pyridine as a copper reagent while omitting the chromogenic reagent. Kwon and Rhee (1986) extracted FFA by isooctane instead of benzene, which omitted the step of solvent evaporation and simplified the determination procedure.

Though the use of copper soap colorimetry in the determination of LA activity is a mature method used in the determination of grain FFA, it is still faced with two limitations. Firstly, the extraction and purification of LA is cumbersome and complex. Secondly, LA is water-soluble while the substrate (such as olive oil) is not and so it is difficult to mix them uniformly. LA only reacts at oil-water interfaces. Due to a great solubility difference of the enzyme solution and substrate in water when mixed, the emulsion within the latex particles has uneven sizes, cannot remain stable and as a result reproducibility is poor. In 2003, Goffman and Bergman extracted LA from rice bran with a phosphate buffered solution. Then the raw LA extract was added to an olive oil emulsion, after 18 h incubation LA activity was determined by copper soap colorimetry. Because of the time-consuming incubation step, this method does not qualify for rapid determination, though it skips the purification of LA. Rose and Pike (2006) developed a method to determine the LA activity of wheat and wheat bran in low-aqueous media. Without extraction or purification of LA, water and substrate were directly added to defatted wheat bran. After incubation with the FFA, the content was determined by copper soap colorimetry and the LA activity determined accordingly. Rose and Pike (2006) discussed how the quantity of water and olive oil affect the LA activity. However, they did not address the following questions: (a) how to control the quantity of water added to the grain samples with different initial water contents; and (b) how to mix uniformly when there was only a little water and olive oil added to the sample, in order to guarantee stable results.

So, this paper takes the lipase of wheat germ as a research object, mainly analyses how the uniformity of mixture, water activity, time of incubation, temperature of incubation and amount of substrate added affect the result of LA activity determination based on the study of the correlation of water activity with water content and finally describes a modified, simple method for determining the activity of wheat germ lipase in low-aqueous media.

2. Materials and methods

Reagents

Oleic acid (C18:1, 99% pure) was purchased from Sigma (Shanghai, China P.R.) and olive oil were from MuelOliva (Zhenjiang, China P.R.). Other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China P.R.). Wheat germ (water content 12.36%) was purchased from Fada Flour Co., Ltd. in Xiajin (China P.R.).

Sample pre-treatment

The method is based on that of Rose and Pike (2006) with modification. Samples of wheat germ flakes, milled from Chinese hard white spring wheat of Fada flour co., Ltd, were blended to provide a uniform sample. A portion of the flakes was ground to pass a 300 μm screen to obtain full fat flour. Ten grams of it were transferred into a conical flask with 100 ml n-hexane. The mixture was oscillated at 180 r/min for 30 min at an air bath temperature shaker THZ-82B (Jintian Medical Equipment Co., Jangsu, China P.R.). Next, the solvent was poured out, the wheat germ transferred onto culture plate and dried at 30 °C in an oven (DHG-9245A, Yiheng science Ltd, Shanghai, China P.R.) for 1 h to drive off the hexane. The defatted wheat germ was stored dry for further use.

Olive oil pre-treatment

Olive oil is a natural oil which contains about 70% oleic acid and 30% other types of long chain fatty acids. It was chosen as the substrate because it allows for quantification of true LA activity (unlike many artificial substrates, such as triacetin). Before using it, the olive oil must be pre-treated and tested to ensure that it was devoid of FFA. The pre-treatment method was based on that of Tiete and Fiereck (1966), with modification. 20% (w/v) alumina was added to the olive oil while stirring once every 10 min for 1 h. The mixture was left standing to deposit alumina. Purified olive oil was prepared after filtering. Five ml of ether and 5 ml of 95% ethanol were added into 5 ml purified olive oil, titrated by 0.05 mol/l NaOH with phenolphthalein as the indicator. If the quantity of NaOH used was more than 0.5 ml, the olive oil had to be purified once more.

Lipase activity determination

There were two groups of centrifuge tubes: one sample (A_f) and one blank (A_i). Each group contained three centrifugal tubes. Four grams partially defatted wheat germ was moved into each tube, 0-2.0 ml distilled water added in and well mixed with the wheat germ and 0.5-5.0 ml of olive oil was added later. An electric homogenate machine (DY89-II, Scientz Biotechnology Co., Ltd, NingBo, China P.R.) was used for further uniformity of the mix.

Twenty ml n-hexane was added into the blank (A_i) and spiral mixed for 30 s, followed by centrifuging at 5,000 rpm/min for 3 min (BR4, Jouan, Winchester, VA, USA). Then n-hexane was decanted from a tube to a round-bottomed flask, repeated for 2 times. The residue was re-dissolved in 4 ml isooctane after vacuum distillation to eliminate the n-hexane. The lidded sample (A_f) was incubated at 30-70 °C for 1.5-9 h.

The oil was extracted in the same way and the rest of the mixture was dissolved in 4 ml isooctane. Two ml 5% (w/v) copper acetate (adjusted to pH 6.1 with pyridine) was added into the two testing tubes respectively. After being shaken vigorously for 1min and centrifuged at 4,000 rpm/min for 3 min, the upper organic phase was transferred into a cuvette and the absorbance value read in a spectrophotometer (UV 9600, Rayleigh, Beijing, China P.R.) at 715 nm. The average of three readings for each test condition was taken.

Lipase activity calculation

The LA activity was calculated according to the following calculation:

$$\text{LA activity} = 1000 \times \frac{(4 + v) \times (A_f - A_i)}{\varepsilon \times t \times l \times s}$$

Where LA activity = lipase activity (U/g); A_f = absorbance of the incubated sample at 715 nm; A_i = absorbance of the blank at 715 nm; ε = molar absorption coefficient ($M^{-1}cm^{-1}$) of oleic acid at 715 nm; l = path length (cm); s = sample dry weight (g); t = incubation time (h); and v = olive oil volume (ml).

Standard curve for oleic acid

Standard oleic acid (dissolved in isooctane) solutions of different concentration 1, 2, 4, 6, 8, 10 mmol/l were prepared. Two ml of copper indicator was added into the 4 ml each of the standard solution described above. The mixture was vigorously shaken for 1 min and centrifuged at 4,000 rpm/min for 3 min and the absorbance of the upper organic phase was determined at 715 nm with isooctane as the reference. The standard curve was drawn according to the averages of 3 determinations for each test condition.

3. Results and discussion

Standard curve for oleic acid

Figure 1 shows the standard curve for oleic acid. The linear regression of absorbance against concentration of oleic acid was Y = 0.11313X + 0.01568 (R^2=0.9992), according to which molar absorption coefficient (ε) at 715 nm was calculated to be 115.3 $M^{-1}cm^{-1}$.

Correlation between water activity and water content

Bell and Labuza (2000) and Greenspan (1977) have found that when the component in solid is certain, water content and water activity has a direct relationship (Bell and Labuza, 2000; Greenspan, 1977). When the water content increased, the water activity increased but in a non-linear manner.

Distilled water additions ranging from 0 ml to 2 ml was added into 4 g partially defatted wheat germ and the mixture

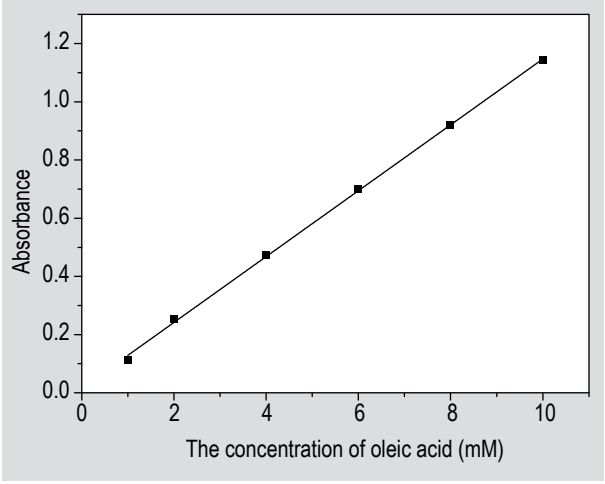

Figure 1. Relationship between the concentration of oleic acid and absorbance value.

was incubated for 24 h. The water content and water activity of the sample were determined, respectively. The correlation of water activity between water content is shown in Figure 2 and there is a clear logarithmic relationship between water activity and water content when the wheat germ water content ranging from 3% to 15%. (Y = 0.324LnX - 0.1958, R^2=0.9954). When the water content was >15%, there is a reduction in the rate of water activity increase and when the water content was >25%, there was little change in water activity.

Effect of water adding amount

The quantity of distilled water added into 4 g partially defatted wheat germ ranged from 0 ml to 2 ml, 2.5 ml olive oil was added and the mixture incubated at 60 °C for 4.5 h. The rest of the testing conditions are consistent with those for the LA activity determination described above.

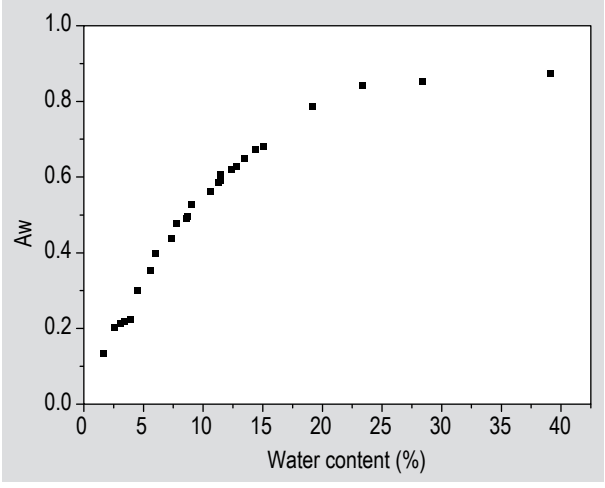

Figure 2. Relationship between water content and water activity (Aw) of wheat germ.

The samples were partially defatted by organic solvent in order to eliminate most of the FFA produced and to activate the lipase in the sample (El Amrani *et al.*, 2003). As shown in Figure 3, the LA activity increases with wheat germ water activity, gradually rising from 0.6 and reaching a peak (3.2 U/g) when the water activity is 0.85. The amount of water added at this point was 1.5 ml/4 g of the sample. LA activity decreased right after the water activity exceeded 0.85. Therefore, the amount of water added was optimized at 1.5 ml. Labuza (1971) suggested that the water activity in food affects enzyme activity significantly that the enzyme had its greatest activity when the water activity reaches approximately 0.8. The result in this study are mostly in agreement with previously recorded studies (Tiete and Fiereck, 1966).

Effect of olive oil adding amount

1.5 ml distilled water was added into 4 g partially defatted wheat germ. The amount of olive oil added ranged from 0 to 5 ml. The mixture was incubated at 60 °C for 4.5 h. The rest of conditions are consistent with those for the lipase activity determination as described above.

The data presented in Figure 4 show that LA activity increased as more oil was added. When the addition was 3 ml, LA activity reached 2.9 U/g. However, with more oil additions the LA activity increase was limited. The probable reason could be that when there is a certain amount of LA, if too much substrate (olive oil) is added, it will reduce the contact opportunities between the water molecules, LA and triglyceride and eventually limit the catalytic activity of LA. So the optimum level of olive oil addition is 3 ml.

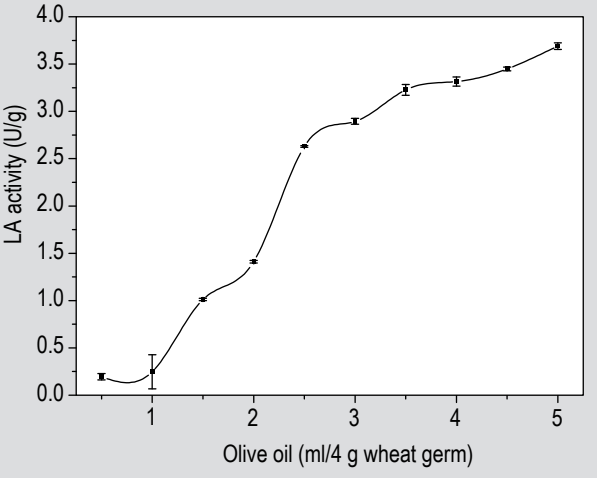

Figure 4. Relationship between the change of wheat germ lipase (LA) activity and the amounts of olive oil. Values are means of three replications. Data are reported as mean value ± standard deviation.

Effect of incubation temperature

1.5 ml distilled water and 2.5 ml olive oil were added into 4 g partially defatted wheat germ and incubated at 30-70 °C for 4.5 h. The rest of the conditions were consistent with those for lipase activity as described above.

As shown in Figure 5, LA activity increases with temperature rise, reaches a peak at about 60 °C and then declines. We chose 55 °C as the best incubation temperature. It has been reported that the optimal temperature of purified LA was 37 °C, which conferred high thermal stability. When incubated at 60-90 °C for 1 h, LA retained 80% of its full

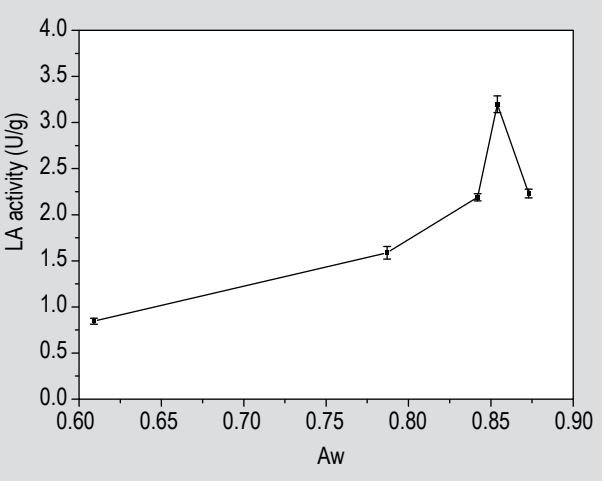

Figure 3. Relationship between wheat germ water activity (Aw) and lipase (LA) activity. Values are means of three replications. Data are reported as mean value ± standard deviation.

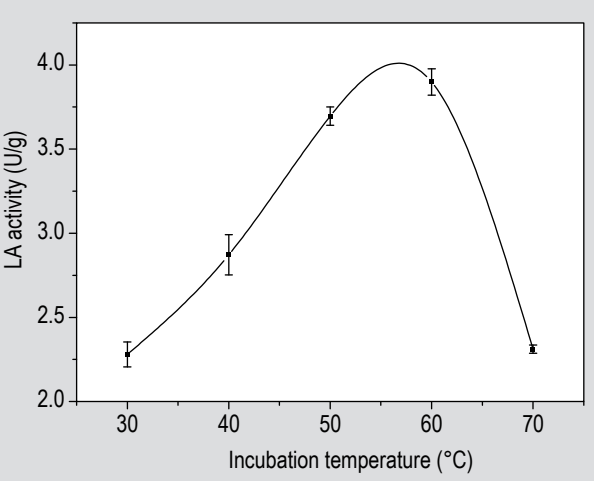

Figure 5. Relationship between the change of wheat germ lipase (LA) activity and incubation temperature. Values are means of three replications. Data are reported as mean value ± standard deviation.

activity (Kapranchikov *et al.*, 2004). Our study is broadly in line with these conclusions.

Effect of incubation time

1.5 ml distilled water and 2.5 ml olive oil were added into 4 g partially defatted wheat germ and incubated at 60 °C for 0-9 h. The rest of conditions were consistent with those for lipase activity as described above.

As shown in Figure 6, wheat germ LA activity reached a maximum after 3-4.5 h incubation and decreases with longer incubation times. We considered 4 h to be the best incubation time.

Precision experiment

1.5 ml distilled water and 3ml olive oil were added into 4 g partially defatted wheat germ and incubated at 55 °C for 4 h. Based on 10 groups of parallel determination, it was calculated that the LA activity was 3.25 U/g and RSD was 5.82% (n=10).

4. Conclusion

In this study we firstly degreased the testing sample with organic solvent to activate LA, which effectively improved the sensitivity of the determination method. Secondly, with a certain amount of distilled water added, LA was set in the most suitable water activity condition, which further increased the sensitivity of the test. Thirdly, to guarantee the reproducibility of results, the sample, olive oil and distilled water were uniformly mixed with an electric homogenizer machine and mortar mill. The proposed method omits the steps of LA extraction and purification, which helps in the rapid determination of activity. The optimized test conditions were:

- Adjustment of wheat germ water activity to 0.85;
- 0.75 ml olive oil added per gram;
- Incubation at 55 °C for 4 h.

Compared with the current method for LA activity determination in grain, this method is simple, rapid and reproducible. It is able to simply and precisely determine LA activity in wheat germ and to enable kinetic studies of enzyme inactivation.

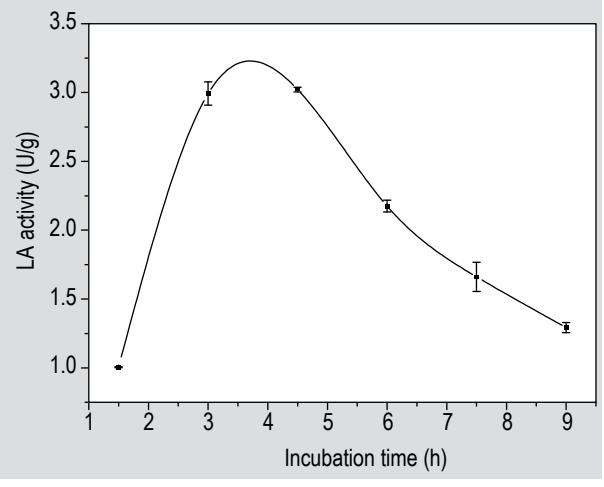

Figure 6. Relationship between the change of wheat germ lipase (LA) activity and incubation time. Values are means of three replications. Data are reported as mean value ± standard deviation.

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References

Baker, D., 1961. A colorimetric method for determining fat acidity in grain. Cereal Chemistry 38: 47-50.

Bell, L.N. and Labuza, T.P., 2000. Moisture sorption: practical aspects of isotherm measurement and use. American Association of Cereal Chemists, St. Paul, MN, USA, 124 pp.

Duncombe, W.G., 1963. The colorimetric micro-determination of long-chain fatty acids. Biochemistry 88: 7-10.

El Amrani, F., Fayol, O., Drapron, R., Potus, J. and Nicolas, J., 2003. Simplified method for determination of lipolytic activity in low moisture media. Sciences des Aliments 23: 209-221.

Goffman, F.D. and Bergman, C., 2003. Hydrolytic degradation of triacyglycerols and changes in fatty acid composition in rice bran during storage. Cereal Chemistry 80: 459-461.

Greenspan, L., 1977. Humidity fixed points of binary saturated aqueous solutions. Journal of Research of the National Bureau of Standards 81: 89-96.

Kapranchikov, V.S., Zherebtsov, N.A. and Popova, T.N., 2004. Purification and characterization of lipase from wheat (*Triticum aestivum* L.) germ. Applied Biochemistry and Microbiology 40: 84-88.

Kwon, D.Y. and Rhee, J.S., 1986. A simple and rapid colorimetric method for determination of free fatty acids for lipase assay. Journal of the American Oil Chemists' Society 63: 89-92.

Labuza, TP., 1971. Kinetics of lipid oxidation in foods. Critical Reviews in Food Technology 2: 355-405.

Lam, H.S. and Proctor, A., 2001. Rapid methods for milled rice surface total lipid and free fatty acid determination. Cereal Chemistry 78: 498-499.

Lowry, R.R. and Tinsley, I.J., 1976. Rapid colorimetric determination of free fatty acids. Journal of the American Oil Chemists' Society 53: 470-472.

Nishiba, Y., Sato, T. and Suda, I., 2000. Convenient method to determine free fatty acid of rice using thin-layer chromatography and flame-ionization detection system. Cereal Chemistry 77: 223-229.

Ramezanzadeh, F.M., Rao, R.M., Windhauser, M., Prinyawiwatkul, W., Tulley, R. and Marshall, W.E., 1999. Prevention of hydrolytic rancidity in rice bran during storage. Journal of Agricultural and Food Chemistry 47: 3050-3052.

Rose, D.J. and Pike, O.A., 2006. A simple method to measure lipase activity in wheat and wheat bran as an estimation of storage quality. Journal of the American Oil Chemists' Society 83: 415- 419.

Tietz, N.W. and Fiereck, E.A., 1966. A specific method for serum lipase determination. Clinica Chimica Acta 13: 352-358.

Xu, B., Miao, W.J., Guo, K., Hu, Q.S., Li, B. and Dong, Y., 2012. An improved method to characterize crude lipoxygenase extract from wheat germ. Quality Assurance and Safety of Crops and Foods 4: 26-32.