Determination of Lipoxidase in Wheat Germ

Jiachun Zhou,[1,2] Xingbo Su,[1] and Xiaoyan Shen[1]

ABSTRACT

Lipoxidase in wheat germ affects the wheat germ's quality and must be inactivated for processing. Because no method was available for measuring unpurified lipoxidase in wheat germ, methods for measuring purified lipoxidase or lipoxidase in soybean were used as reference methods. These methods proved to be so inaccurate that the natural state of the wheat germ could not be determined. A simple method was therefore established to determine lipoxidase in wheat germ. It includes enzyme extraction, substrate preparation, and conditions for termination of the enzymatic action. Furthermore, the optimum inactivation conditions for lipoxidase in wheat germ were tested.

Wheat germ protein is a complete protein that accounts for as much as 30% of the total essential amino acid in wheat (3,6). Wheat germ is ≥10% oil, 84% of which is unsaturated fatty acid. In addition, wheat germ contains many other components such as phospholipids, glutathion, tocopherol, octacosanol, and flavone (2). Wheat germ is difficult to store because it oxidizes and rancidifies in a few days. Lipoxidase in wheat germ is considered to be the main factor because it can catalyze oxidation of wheat germ oil to cause a rapid increase in the peroxide value (1,4).

A proper method for measuring lipoxidase activity in natural wheat germ is a prerequisite for determining how to inactivate it. One published method for measuring purified lipoxidase activity (5) did not properly stop the enzymatic action, and it required rigid constant temperature control the whole time. A method for measuring soybean lipoxidase activity (7) was also unsuitable for wheat germ because, although soybean lipoxidase was inactivated by the method, wheat germ lipoxidase remained high, causing a large deviation in measurement. These two methods are not good references for measuring lipoxidase activity in natural wheat germ. Consequently, an alternative method was developed to overcome these shortcomings.

[1] Institute of Biochemical Engineering, East China University of Science and Technology, Shanghai 200237.

[2] Corresponding author. Phone/Fax: +86-21-64253064, E-mail: jczhou@ecust.edu.cn.

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MATERIALS AND METHODS

Materials and Instruments

Fresh wheat germ was obtained from Shanghai Flour Company Ltd. Linoleic acid, with 99% purity, was Fluka-62230, and Tween 20 was Fluka-93773. The UV/Vis spectrophotometer, model UV-2100PC, was obtained from UNICO (Shanghai) Instruments Co., Ltd. A Precisa electronic balance model XS225A, a homogenizer (model SilentCrusher) from S. Heidolph (Germany), and a centrifuge, model DL-5B, from Shanghai Centrifuge Research Institute were used.

Measurement of Lipoxidase in Wheat Germ

Defatted wheat germ extract (0.3 mL) with proper concentration was added to 2 mL of boric acid buffer solution, pH 9.0, containing linoleic acid at 2.24×10^{-3} mol/L. Both extract and buffer were kept at 30°C before mixing. The mixture was held for 4 min at 30°C, and then the enzymatic action was terminated by adding 2 mL of 1.5 mol/L NaOH. The optical absorption was detected at 234 nm.

Definition of the Activity Unit of Lipoxidase in Wheat Germ

In the above-mentioned lipoxidase measuring system, an increase of 0.001 in optical absorption caused by 1 g of wheat germ per minute was defined as 1 enzyme activity unit (U).

RESULTS AND DISCUSSION

Determination of lipoxidase activity in wheat germ is influenced by several factors: the defatting of the wheat germ, the extraction of lipoxidase, the concentration of the extract, and especially the method for stopping the lipoxidase reaction.

Defatting of Wheat Germ

If the wheat germ were not defatted, the linoleic acid existing in it would be catalyzed by lipoxidase during the extraction of lipoxidase, which would cause high optical absorption in the extract and make measurement of the enzyme activity impossible. The fats in wheat germ must be removed thoroughly, but enzyme activity must not be affected during the process. An appropriate method was found after many experiments. The wheat germ was crushed and filtered through a 100-mesh sieve. This wheat germ powder was then soaked in petroleum ether for 12–14 hr at 30°C. The amount of petroleum ether used was three times the wheat germ's weight. The defatted wheat germ was filtered and evaporated at room temperature to remove residual petroleum ether.

Extraction of Lipoxidase from Wheat Germ

To extract lipoxidase from wheat germ, a suspension of 1 g of defatted wheat germ powder in water was shaken for 30 min at 30°C and centrifuged for 20 min at 5,000 rpm to a certain volume; this was used as the enzyme solution. The effect of concentration of the lipoxidase extract on the measurement of enzyme activity is shown in Table I, where increased enzyme activity correlates with increased absorption.

When 1 g of enzyme extract was diluted to 10 mL, the enzyme reaction system had higher protein concentration and lower transparency, and the optical absorption value did not show an obvious change. When the enzyme extract was diluted to 50 or 100 mL, the optical absorption value changed relatively obviously. To analyze the activity of enzyme that had been partly inactivated by a heat treatment, the enzyme extract was diluted to 50 mL. Generally speaking, the addition of a homogenization step before shaking for 30 min was helpful for protein extraction. The effect of homogenization on the wheat germ extract's lipoxidase activity is shown in Table II.

Table II shows that enzyme activity peaked immediately and remained constant in samples homogenized for 90 sec, when tested at two dilutions. This might mean that wheat germ lipoxidase tended to be inactivated by the shear force at high speed in the water solution. But the crushing of wheat germ did not affect the enzyme activity, because defatted wheat germ that passed through 60-, 80-, and 100-mesh had almost the same enzymatic activity. It was reasonable for us to suspect that the observed reaction profile was the direct result of improved protein extraction with homogenization. Did the higher protein concentration mask the increase of optical absorption? We diluted the homogenized extracts to half the original concentration; that is, 1 g of enzyme extract was diluted to 100 mL. The results were shown in Table III. It shows that protein concentration was not the main factor.

Substrate Preparation

The substrate system for measuring wheat germ lipoxidase activity was a boric acid buffer solution, pH 9.0, containing linoleic acid at 2.24×10^{-3} mol/L. In order to disperse linoleic acid into the buffer solution, 0.05% of Tween 20 was required.

Conditions for Termination of the Wheat Germ Lipoxidase Reaction

For enzyme activity analysis, the enzymatic action needed to be stopped immediately when necessary. The method for terminating soybean lipoxidase reaction was to add anhydrous ethanol at a volume twice as high as that of the reaction system. However, for wheat germ lipoxidase, the optical absorption value kept increasing continuously even when five times the amount of anhydrous ethanol was added. Moreover, because ethanol diluted the peroxidate produced, the optical absorption was greatly reduced, and some discrepancy could be concealed.

Denaturation by heat is a common method for enzyme deactivation. The activity of soybean lipoxidase could be inhibited when the temperature was higher than 85°C, but the activity of wheat germ lipoxidase remained comparatively high even when heating lasted 2 min at 95°C. Even if the lipoxidase extract was heated for 3 min in boiling water, the enzymatic activity could still occur.

Much work was done to find proper termination conditions for the wheat germ lipoxidase reaction. It was found that enzymatic action could be stopped by adding 2 mL of 1.5 mol/L NaOH. Acids, organic solvents, and many kinds of metal ions were tested for the same purpose, but none could terminate the reaction effectively. A concentration of NaOH lower than 1.5 mol/L could not inactivate wheat germ lipoxidase; a higher concentration of NaOH destroyed the emulsified substrate and made it opaque so that it could not be analyzed.

Table I. Optical absorption (at A_{234}) at different concentrations of lipoxidase extract

Supernatant	Enzymatic Action Time (min)								
Diluted to (mL)	5	10	15	20	25	30	40	50	60
10	1.209	1.209	1.209	1.213	1.213	1.213	1.213	1.215	1.215
50	0.934	0.943	0.952	0.961	0.970	0.981	0.990	1.002	1.015
100	0.625	0.632	0.637	0.646	0.655	0.665	0.675	0.684	0.694

Table II. Effect of homogenization time on activity (optical absorption at A_{234}) of lipoxidase extract from wheat germ (2% dilution)[a]

Homogenization	Enzymatic Action Time (min)								
Time (sec)	5	10	15	20	25	30	40	50	60
0	0.755	0.759	0.762	0.766	0.770	0.775	0.785	0.796	0.810
5	0.878	0.879	0.880	0.882	0.884	0.887	0.892	0.899	0.907
10	1.013	1.014	1.015	1.017	1.020	1.021	1.025	1.030	1.033
20	1.081	1.082	1.082	1.084	1.085	1.087	1.091	1.095	1.110
30	1.072	1.072	1.074	1.074	1.075	1.075	1.077	1.078	1.079
40	1.077	1.077	1.079	1.080	1.080	1.081	1.081	1.082	1.082
60	1.097	1.098	1.098	1.100	1.100	1.100	1.101	1.101	1.101
90	1.087	1.087	1.087	1.087	1.087	1.087	1.087	1.087	1.087
120	1.079	1.079	1.079	1.079	1.079	1.079	1.079	1.079	1.079
180	1.065	1.065	1.065	1.065	1.065	1.065	1.065	1.065	1.065

[a] Dilution: 1 g of enzyme extract was diluted to 50 mL.

Table III. Effect of homogenization time on activity (optical absorption at A_{234}) of lipoxidase extract from wheat germ (1% dilution)[a]

Homogenization	Enzymatic Action Time (min)								
Time (sec)	5	10	15	20	25	30	40	50	60
0	0.448	0.450	0.452	0.453	0.454	0.457	0.459	0.461	0.464
5	0.547	0.548	0.549	0.550	0.552	0.553	0.557	0.560	0.566
10	0.616	0.617	0.618	0.619	0.619	0.620	0.622	0.623	0.625
20	0.664	0.664	0.665	0.666	0.666	0.667	0.667	0.667	0.668
30	0.660	0.660	0.661	0.661	0.661	0.662	0.662	0.663	0.663
40	0.665	0.665	0.667	0.667	0.667	0.669	0.669	0.669	0.670
60	0.672	0.672	0.672	0.672	0.672	0.672	0.672	0.672	0.672
90	0.668	0.668	0.668	0.668	0.668	0.668	0.668	0.668	0.668
120	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685
180	0.632	0.632	0.632	0.632	0.632	0.632	0.632	0.632	0.632

[a] Dilution: 1 g of enzyme extract was diluted to 100 mL.

Linear Area for Wheat Germ Lipoxidase Activity Measurement

The optical absorption at A_{234} of wheat germ lipoxidase, under the enzymatic conditions described, was 0.052 at 1 min, 0.137 at 2 min, 0.237 at 3 min, 0.339 at 4 min, 0.406 at 5 min, 0.469 at 6 min, 0.518 at 7 min, and 0.562 at 8 min.

The enzymatic action of wheat germ lipoxidase was linear within 5 min, and can be described by the equation

$$y = 0.091x - 0.0388 \quad R^2 = 0.9959$$

The slope of this equation was 0.091, which means that A_{234} increased by 0.091 per minute. Because 1 g of wheat germ extract was diluted to 50 mL, according to the definition of activity unit, the activity (A) of the wheat germ lipoxidase tested was

$$A = 0.091 \times 1{,}000 \times 50 = 4{,}550 \text{ U}$$

Inactivation of Lipoxidase in Wheat Germ by Heat Treatment

Natural wheat germ lipoxidase was so thermotolerant that it was hard to inactivate. It is important to find a suitable heating condition under which lipoxidase can be inactivated. When wheat germ was heated from 110 to 140°C, the percentage of lipoxidase activity retained in the wheat germ decreased (Figs. 1–4).

Because wheat germ tended to burn when it was heated at more than 140°C, the proper heating conditions for inactivating wheat germ lipoxidase were determined to be 10 min at 130°C.

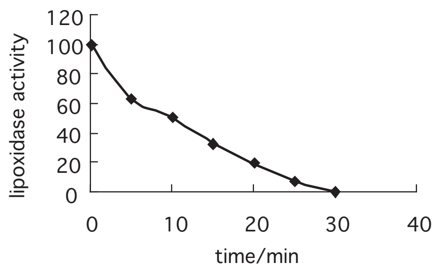

Fig. 1. Lipoxidase activity retained in wheat germ heated to 110°C.

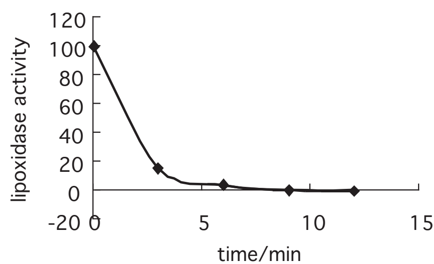

Fig. 3. Lipoxidase activity retained in wheat germ heated to 130°C.

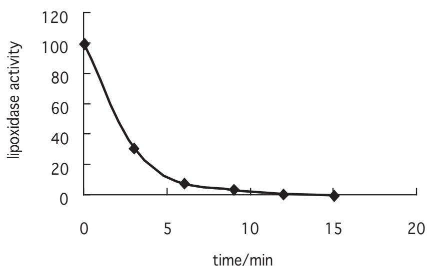

Fig. 2. Lipoxidase activity retained in wheat germ heated to 120°C.

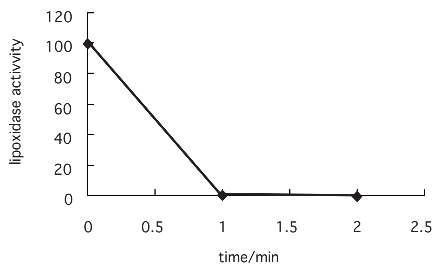

Fig. 4. Lipoxidase activity retained in wheat germ heated to 140°C.

CONCLUSION

A method of measuring lipoxidase in natural wheat germ was developed that included a critical step involving the homogenization of the raw wheat germ for 90 sec to improve enzyme extraction.

Termination of the wheat germ-lipoxidase activity was achieved by mixing NaOH and enzyme reaction solution at a ratio of 2 mL of NaOH (1.5 mol/L) to 2.3 mL of solution.

Wheat germ lipoxidase was inactivated by heating the raw wheat germ to 130°C and holding for 10 min.

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