A Novel Method to Assay Peroxidase Activity

J. Liu, Y. Zhang, L. Qiu, Y. Xia, W. He, and J. Cheng
P.O.Box 70, Qingdao University of Science and Technology,
Zhengzhou Road 53, Qingdao, Shandong, P. R.China, liujhye@sina.com.cn

Original scientific paper
Received: December 12, 2003
Approved: December 15, 2004

A novel method to assay soybean hull peroxidase (SHP) activity was developed in which aniline was used as substrate. The method is different from some existing methods on substrate. Solution of ethanol and water was used as a solvent in which aniline is soluble, and 1 : 8 : 36 ratio of aniline-ethanol-peroxidase solution was optimal for determining. The enzymatic reaction occurs in a solution and is a homogeneous phase reaction. Other proper condition employed was as follows: the range of the volume fraction of H_2O_2 from 2.0×10^{-5} to 4.0×10^{-5}; the volume fraction of aniline from 1 % – 2.5 %; pH, 5.5; the range of temperature, 18 – 30 °C, respectively. Under the condition, both the accuracy and precision of the method are satisfactory.

Key words:
Peroxidase; aniline; enzyme activity

Introduction

Peroxidases (EC1.11.1.7) are widespread in biosphere and are capable of catalyzing a variety of organisms or inorganisms in the presence of hydrogen peroxide or organic peroxide.[1] Peroxidase can be used in many applications. The information from Internet has shown that SHP has been widely used in food, medicine and environment protection, and chemical industry. From the present practical applications we can draw a conclusion, that SHP has been an extremely conceited enzyme in the same kind of enzymes because of its unique stability and excellent reactiveness. For example, in USA SHP has been employed in treatment of industrial wastewater by environment protecting departments; American Enzymol International and American Qualax Company use SHP in the production of reagent box which is used in medical diagnose. The function and quality of the products all have advantages over traditional HRP-test box. In resin production industry, SHP successfully replaces the formaldehyde made from conventional synthesis industry, reducing the cost to a great extent. In addition, many large companies actively develop using SHP in the production of computer chips, varieties of plastics and agglutinant for construction materials.[2] At present, the existing methods of assaying peroxidase activity, such as RZ value methodology, guaiacol method and pyrogallol acid method are already very available, but the determining reactions almost occur in microemulasions and these methods are more inconvenient to be used widely. On the other hand, substrates (such as ABTS, 2,6-dimethoxyphenol or 4-aminoantipyrine) used in other methods are very expensive. Thus these methods are not the perfect ones; therefore, some improvement should be made. According to the nature of the peroxidase, aniline was used as substrate in our work. In the presence of H_2O_2, peroxidase catalyzed the oxidation of aniline, and a polymer of aniline was then produced. With this reaction, peroxidase activity is determined.

Materials, apparatus and methods

Materials

Soybean hulls were purchased from a market of agricultural products. Aniline, acetone, ethanol, hydrochloric acid, sodium hydroxide, hydrogen peroxide (30 % solution in water) were commercially available and were used without further purification.

Apparatus

High speed Tissue Triturator DS–1 was purchased from Shanghai Sample Model Manufactory (Shanghai, P.R.China). S54 Spectrophotometer was obtained from Lengguang Technique Co., Ltd. (Shanghai, P.R.China). High Speed Refrigerated Centrifuge GL–21M was from Xiangyi Centrifuge INSTRUMENT Co., Ltd. (Changsha, Hunan, P.R.China).

Methods

Preparation of peroxidase solution

First, dry soybean hulls hardly containing the fragments of soybean meat were prepared. 40 g of

*Corresponding author. e-mail: liujhye@sina.com.cn

soybean hulls were soaked in 280 ml of deionized water for 2 h. Then the soaked soybean hulls were transferred into a high-speed tissue triturator with 200 ml of deionized water in it. The hulls were homogenized at 12 000 rpm for 1.5 min, and then, the homogenate was extracted for 12 h at temperature of 4 °C. Finally, the homogenate was filtered to remove the larger particles with cheesecloth. The filtered homogenate was centrifuged at 12 000 rpm for 10 min. The supernatant obtained was saved at 4 °C and used as peroxidase solution or original peroxidase solution required.

Dissolvability of aniline, and selection of solvent

Aniline is freely soluble in ethanol and insoluble in water; therefore, mixture solution of ethanol and water was used as a solvent in our assaying. To prepare testing solution, aniline-ethanol solution with certain volume fraction was prepared and then it was added into peroxidase solution to get ethanol-aniline-peroxidase solution. Aniline-ethanol solutions with different volume fractions were prepared: φ = 50.0 %, 33.3 %, 25.0 %, 20.0 %, 16.6 %, 14.4 %, 12.5 % and 11.1 %, respectively. When 2 ml of aniline-ethanol solution was added to 8 ml of peroxidase solution mentioned above, the volume fractions of ethanol of 8 aniline-ethanol-water solutions were as follows: φ = 10.0 %, 13.34 %, 15.0 %, 16.0 %, 16.68 %, 17.12 %, 17.5 %, 17.78 %, respectively while the volume fractions of aniline of 8 aniline-ethanol-water solutions were: φ = 10.0 %, 6.66 %, 5.0 %, 4.0 %, 3.32 %, 2.88 %, 2.52 %, 2.22 %, respectively. The dissolvability of aniline in ethanol solution was observed with a microscope and the dissolution time was also recorded.

Selection of optimum absorbance wavelength

80 μl of peroxidase solution taken by micro-sample injector were added into a glass bottle. To dilute the peroxidase solution, 8 ml of deionized water are required. Then 2 ml of aniline-ethanol solution (φ = 11.1% of aniline) was added into the bottle. 0.1ml of H_2O_2 (φ = 1%) was added into above solution in which peroxidase catalyzed the oxidation of aniline in the presence of H_2O_2. After 1 h, the reacted solution was scanned by spectrophotometer with control solution (without H_2O_2 in it) as a blank.

The relation of enzyme concentration and activity

The peroxidase solution was diluted into 5 samples with different percentages of the starting fraction: 2 %, 1.3 %, 1 %, 0.8 %, and 0.67 %. 2 ml of aniline-ethanol solution (φ = 11.1 %) and 100 ml of H_2O_2 (φ = 1 %) were added into each dilution, corresponding to final volume fraction of resulted solution of H_2O_2, 4×10^{-5}, and aniline, 2.22 %, and the solution was mixed quickly. The mixture (3 ml) was placed in a spectrophotometer quartz cuvette of a spectrophotometer and absorbance against deionized water was determined at 415 nm. The absorbance values were recorded every 30 s. Under the conditions, the absorbance was determined two more times. One unit of peroxidase activity (U) represents the change of absorbance value in 1 min at room temperature (1cm quartz cuvette is used in the definition).

Effect of the volume fraction of aniline on the peroxidase activity

Under the conditions of superfluous substrate, aniline-ethanol-peroxidase solution was prepared by mixing aniline-ethanol solution with diluted enzyme solution. With a given fraction of peroxidase, the volume fraction of aniline was changed as follows: φ = 4.4 %, 3.3 %, 2.2 %, 1.1 %, and 0.55 %, respectively. 10 ml of aniline-ethanol-peroxidase solutions with above volume fractions of aniline were prepared respectively and H_2O_2 (φ = 1 %) was added into five solutions above to make the final volume fraction of H_2O_2 up to 4.0×10^{-5}, respectively. After the solution was mixed quickly, the absorbance of each solution was determined. Five solutions to test were obtained as follows: (1) 4 ml aniline-ethanol solution mixed with 1ml of deionized water; (2) 3 ml aniline-ethanol one, with 2 ml of deionized water; (3) 2 ml aniline-ethanol one, with 3 ml of deionized water; (4) 1 ml aniline-ethanol one, with 4 ml of deionized water; (5) 0.5 ml aniline-ethanol one, with 4.5 ml of deionized water, respectively. 5 ml of the diluted solution (1 % of the original peroxidase solution) and 100 μl H_2O_2 (w = 1 %) were added into above five solutions (corresponding to a volume fraction of H_2O_2, 4.0×10^{-5}), respectively. After the solution was mixed quickly, the absorbance was determined, respectively.

Effect of the volume fraction of H_2O_2 on the peroxidase activity

With φ = 2.2 % of aniline and a given concentration of peroxidase, the effect of the volume fraction of H_2O_2 on the peroxidase activity was determined. The volume fraction of H_2O_2 was altered as follows: 0.5×10^{-5}, 1.0×10^{-5}, 2.0×10^{-5}, 4.0×10^{-5}, 10.0×10^{-5}, 15.0×10^{-5} and 20.0×10^{-5}, respectively. After the solution was mixed quickly, the absorbance was determined, respectively.

Effect of pH on the peroxidase activity

0.5 ml of the original solution of peroxidase was respectively added into each of flasks. With some water added into above 4 flasks, pH of each solution was adjusted in turn with hydrochloric acid

to near to 4, 5, 6 and 7. After diluting each solution with deionized water to the scale of the flask, pH of each solution was measured with a pH meter. Absorbance of every solution was measured as mentioned above while the volume fraction of H_2O_2 was kept at 2.0×10^{-5}.

Effect of temperature on the peroxidase activity

2 ml of aniline-ethanol solution (φ = 11.1 %) was added into 8 ml of diluted enzyme solution (1 % of the original concentration) to form a mixture. The temperature of each run was controlled at approximately 15 °C, 20 °C, 25 °C and 30 °C, respectively. Absorbance at each temperature was measured at 415 nm for 5 min. It was necessary that the sample and reference cuvettes were adjusted to the same temperature while the absorbance at 415 nm was measured for 5 min. Every run was repeated three times.

Repetition experiment

3 solutions were prepared by diluting 0.5 ml of enzyme solution to 1 % of the original fraction and adjusting each pH near to 6.0. Above 3 solutions it was referred to as 1# (pH 6.00), 2# (pH 6.01) and 3# (pH 6.02), respectively. In another day, the other three solutions were prepared as mentioned above and recorded as 4# (pH 6.0), 5# (pH 6.0) and 6# (pH 6.0). Absorbance of each solution was measured in same day.

Results and discussion

Dissolvability of aniline, and selection of solvent

Aniline molecule is a polar one, but it is difficult for aniline to dissolve in water. To make enzymatic reaction occur in solution, aniline was dissolved in ethanol, and this resulted in the increase of the solubility of aniline. In order to decrease the inhibition of ethanol on peroxidase, lower volume fraction of ethanol is required. Moreover, aniline was completely soluble in the mixture solution of ethanol and peroxidase solution only in certain range of volume fraction of ethanol. Thus it is necessary to determine the range through a test. At this volume the fraction of ethanol mixture of aniline, ethanol and peroxidase solution is not microemulasions but solution. The enzymatic reaction occurs in solution and is a homogeneous phase reaction, whereas when the reaction occurs in microemulasions, it is an interfacial reaction, e.g. heterogeneous phase reaction. For this reaction in our study, the homogeneous reaction obviously excels the interfacial one.

Moreover, in our study we have observed that polymer produced in the reaction was soluble easily in ethanol. Consequently the polymer would not deposit on the wall of the cuvette. Table 1 shows that dissolvability of aniline in the ethanol solution, in which the volume fraction of ethanol and aniline was 22.23 % and 2.22 %, respectively, is the best in 8 mixture solutions. Hence, the solution with a volume fraction of 2.22 % aniline was used in our study.

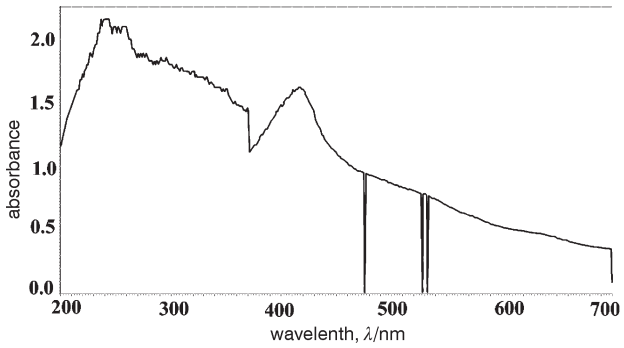
Additionally, acetone was ever used as a solvent in our study. But the polymer had a poor solubility in the acetone – peroxidase solution, which resulted in the sediment of polymer on the wall of cuvette, and the part (%) of light penetration was influenced by the sediment of polymer, so that the accuracy of measurement decreased. In the end, acetone was abandoned as a solvent; therefore, only ethanol was used as a solvent throughout our tests.

Table 1 – *Dissolvability of aniline in the solution of ethanol-water*

Volume fraction of aniline	Dissolution state	Time of dissolution t	Volume of ethanol fraction φ	Remark
10.0 %	prodigious oil drops, milk-white precipitate	over 9 min	10.0 %	Dissolution states of the solutions with aniline
6.66 %	tiny oil drops, milk-white precipitate (with little brown)	over 6 min	13.34 %	concentration of 3.60 %, 3.13 % and 2.22 %,
5.00 %	minute oil drops, two times more clarified than former, but with little Milk-white precipitate	over 8 min	15.0 %	were observed by microscope, respectively.
4.00 %	macroscopical clarification	dissolve within 4 min	16.0 %	We found that 3.60 %,
3.32 %	macroscopical clarification	dissolve within 3 min	16.68 %	3.13 % solutions still
2.88 %	macroscopical clarification	dissolve within 3 min	17.12 %	had a little oil drops
2.52 %	macroscopical clarification	dissolve within 3 min	17.50 %	but 2.22 % solution
2.22 %	macroscopical clarification	dissolve within 3 min	17.78 %	had a complete ddissolution.

Selection of optimum absorbance wavelength

From Fig. 1 we can see that reacted solution has significant absorbance at 240 nm and 415 nm. However, the composition of reaction solution is complex, including protein, nucleic acid etc. These substances all have absorbance in the ultraviolet region. The absorbance peak at 240 nm may be produced by above substances. The reaction solution is colourless before reaction occurs. But the product is brown. According to this fact, we can conclude, that the contaminants in reaction solution cannot interfere with the absorbance of reaction solution. *Liu*[1] et al. have testified that haemachrome coradical contained in the soybean hull peroxidase has absorbance at 406 nm. But the enzyme solution was diluted in our study; enzyme concentration was so little that the interference made by the contaminants could be ignored. As a result, 415 nm was regarded as the optimum wavelength at which the absorbance was determined in our work.



vertical coordinate: Absorbance
horizontal coordinate: Wavelength (nm)

Fig. 1 – *Spectrum of the reacted solution*

Relation of enzyme concentration and enzyme activity

According to Fig. 2, the linearly dependent coefficient between enzyme concentration and enzyme activity, is 0.9958. Thus, it may be concluded that the relation between enzyme concentration and activity is linear under the condition employed. In the catalysis, if the substrate concentration is great enough to saturate the enzyme, rate of the enzymatic reaction (which is expressed through the rate of change of absorbance) is positive to enzyme concentration. Therefore, enzyme activity can be denoted using the rate of change of absorbance, and can be further used to express enzyme concentration. Because the polymer molecule only have molecular weight disturbing, we can presume from the experiment that rate of change of absorbance is probably positive to the chemical bond (produced by the monomers of polymers) concentration.

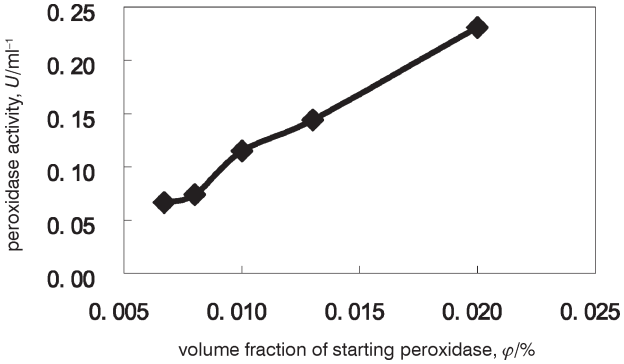


Fig. 2 – *Peroxdase volume fraction vs. its activity*

Effect of hydrogen peroxide concentration on the enzyme activity

The curve depicted in Fig. 3 shows the effect of H_2O_2 volume fraction on the peroxidase activity. The inhibition effect of H_2O_2 on peroxidase is referred to as substrate inhibition. It can be observed from Fig. 3 that enzyme activity is the highest at the volume fraction of H_2O_2, $2.0X10^{-5}$. But higher volume fraction of H_2O_2 is required in order to ensure the saturation of H_2O_2 to all peroxidase during the test reaction, when the concentration of peroxidase is higher. Analysis of Fig. 3 shows that a range of the volume fraction of H_2O_2 from $\varphi = 2.0 \times 10^{-5}$ to 4.0×10^{-5}, is favorable for measurement of the absorbance.

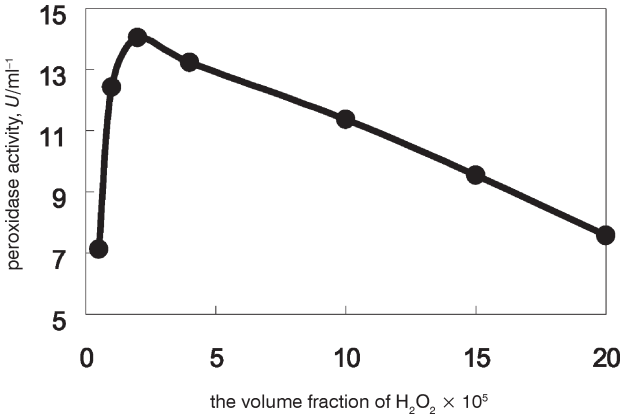


Fig. 3 – *Effect of the volume fraction of H_2O_2 on the peroxidase activity*

The mechanism of peroxidase catalyzing the oxidation of aromatic substrates in presence of hydrogen peroxide is usually depicted by the Chance--George mechanism:[3]

$$E + H_2O_2 \rightarrow E_1 + H_2O \quad (1)$$

$$E_1 + AH_2 \rightarrow E_2 + AH \quad (2)$$

$$E_2 + AH_2 \rightarrow E + AH + H_2O \quad (3)$$

The native enzyme (E) is oxidized by hydrogen peroxide to an active intermediate enzymatic form called compound I (E_1), which caries out the oxidation of an aromatic compound (AH_2) molecule into a free radical ($AH^{\cdot}$), whereas the enzyme turns into compound II (E_2). Compound II oxidizes a second aromatic molecule that produces a second radical and returns the enzyme to its native state, thereby completing the cycle. The overall reaction is as follows:

$$H_2O_2 + 2\,AH_2 \rightarrow 2\,AH + 2\,H_2O \qquad (4)$$

Free radicals, formed during the cycle diffuse, change the enzyme into the bulk solution where they react to form polyermartic products. Reaction (4) indicates that two free radicals are generated for every molecule of peroxide.

While the reactions represented by Eq. (4) dominate in the assay mixture, a number of side reactions also occur. Some are responsible for inactivation of peroxidase. A significant quantity of compound II can be oxidized by hydrogen peroxide to the compound III (E_3) state according to the following scheme:[4–5]

$$E_2 + H_2O_2 \rightarrow E_3 + H_2O \qquad (5)$$

Compound III is not catalytically active but its formation does not represent a terminal inactivation of SHP since it decomposes spontaneously to native peroxidase according to:[6]

$$E_3 \rightarrow E + O_2^- + H^+ \qquad (6)$$

Once in the compound III form, the enzyme is hampered in carrying out the catalytic oxidation of aromatic substrate since the return to native enzyme form is very slow.

The curve in Fig. 3 indicates that at low hydrogen peroxide concentrations, low activity is due to the largest fraction of peroxide existing in the native state. Therefore, the lack of hydrogen peroxide limits the conversion of native enzyme to compound I and II which are responsible for polymer products formation. At high hydrogen peroxide concentrations, the native enzyme concentration decreases significantly, but the formation of compound III outweighs all other enzyme forms, thereby producing a covered rate of polymer products formation.

Effect of volume fraction of aniline on the enzyme activity

The effect of volume fraction of aniline on enzyme activity is shown in Fig. 4. A high enzyme activity was observed at the volume fraction of aniline about 0.015. But a low activity appears in the figure when the volume fraction of aniline is less than 0.09, aniline is consumed very soon so that catalysis is over after lessen time. This makes measured enzyme activity lesser and the error larger. When the volume fraction of aniline is more than 2.4 %, enzyme activity becomes lesser instead. This is resulted from substrate inhibition that is similar to the inhibition of H_2O_2 on peroxidase activity. So there is an optimum volume fraction range in which aniline has not obvious inhibition on the enzyme activity. Hence, to make the enzyme activity up to the highest, 1.8 % of aniline volume fraction is required. The results shown in Fig. 4 suggest that an optimum enzyme activity can be obtained when the volume fraction of aniline in reaction solution is in the range from 1 % – 2.4 %.

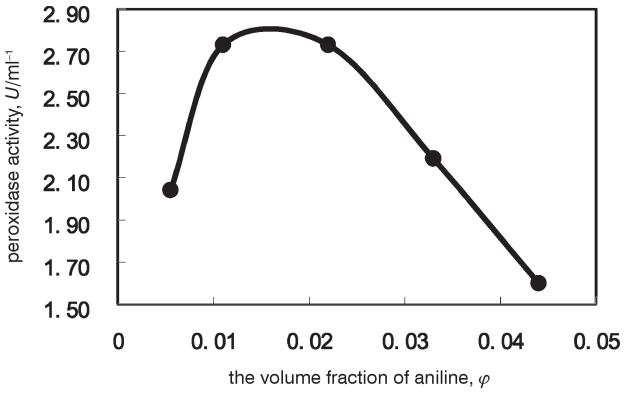


Fig. 4 – *Effect of the volume fraction of aniline on the peroxidase activity*

Effect of temperature and pH on the enzyme activity

Shown in Fig. 5, the optimum pH of the peroxidase was about 5.5, and the effect of pH on the activity was considerable. It was necessary to keep an accurate pH for determining the activity of peroxidase in the method.

Fig. 6. shows the activity vs. temperature. Seen from the same Figure, the activity increased with

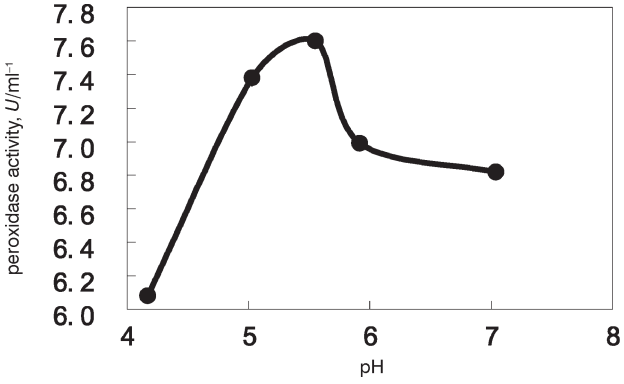


Fig. 5 – *Effect of pH on the peroxidase activity*

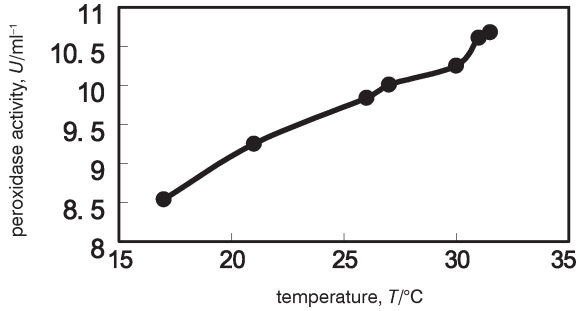


Fig. 6 – *Effect of temperature on the Peroxidase Activity*

the increase of temperature. The range of temperature employed was 17 – 31.5 °C which was covered with most of room temperature. The enzyme showed higher activity at higher temperature, as was consistent with another report[7] that showed that the peroxidase was stable at higher temperature.

Repetition experiment

Analysis of variance (T Test, $\alpha = 0.1$) shows that this method to determine the peroxidase activity is dependable (Table 2, Table 3). The repetition test confirms that the accuracy and precision of the method are both higher.

Table 2 – *Data of repetition experiment*

No	1	2	3	4	5	6	Average peroxidase activity, *U* ml-1
1#	9.61	9.46	9.88	10.02	10.12	10.2	9.88
2#	10.31	10.13	10.61	10.77	10.58	10.43	10.47
3#	11.14	10.71	11.08	10.86	11.00	10.68	10.91
4#	9.85	9.91	9.59	9.48			9.71
5#	10.35	10.60	9.65	9.69	10.03	9.99	10.05
6#	10.98	10.8	10.48	10.36			10.66

Conclusions

Soybean hull peroxidase (SHP) activity was assayed with a novel method in which aniline was used as a substrate. Solution of ethanol and water was used as a solvent in which aniline was soluble, and 1 : 8 : 36 ratio of aniline: ethanol: peroxidase solution was optimal for determining the activity. The enzymatic reaction occured in a solution and was a homogeneous phase reaction. Other proper

Table 3 – *Variance analysis (T test)*

1#	2#	3#	4#	5#	6#
1#	1.62	2.31	2.03	0.62	1.05
2#		1.43	1.25	0.38	0.65
3#			0.88	0.31	0.46
4#				0.31	0.52
5#					1.68
$F_{0.90}(5, 5)$ 3.45		$F_{0.90}(5, 3)$ 5.31		$F_{0.90}(3, 5)$ 3.62	
$F_{0.10}(5, 5)$ 0.29		$F_{0.10}(5, 3)$ 0.28		$F_{0.10}(3, 5)$ 0.19	

condition employed was as follows: the range of the volume fraction of H_2O_2 from $\varphi = 2.0 \times 10^{-5}$ to 4.0×10^{-5}; the volume fraction of aniline from φ = 1 % – 2.5 %; pH, 5.5, the range of temperature, 18–30 °C, respectively. Under the condition, both, the accuracy and precision of the method developed are satisfactory.

Additionally, reaction in this method is homogeneous phase one and it has advantage over the classic microemulasions reaction in which the reaction is heterogeneous phase one. The substrate, aniline, used in this test is cheap and available. Thus the method used in our work is advantageous over the existing methods. And it may be widely used in measuring the peroxidase activity, and the method is dependable.

ACKNOWLEDGMENT

The project was supported financially by the National Science Foundation of China (Grant No.20176019)

References

1. *Liu Wen, Fang Jing*, Chinese biochemistry and molecular biology journal, **11**(5) (1998) 577.
2. *Liu Wen, Fang Jing, Gao Pei-Ji.* Science and Technology of cellulose (Chinese) **8** (2) (2000) 60.
3. *Job, D., Dunford, H. B.,* Eur. J. Biochem. **66** (1976) 607.
4. *Adediran, A., Lambeir, A.* Eur. J. Biochem. **186** (1989) 571.
5. *Nakajima, R., Yamazaki, I.,* J.Biol.Chem. **262** (1987) 2576.
6. *Arnao, M. B., Acosta, M., del Rio, J. A., Varon, R., Garcia Canovas, F.* Biochim. Biophys. Acta **1041** (1990) 43.
7. *Henriksen, A., Mirza, O., Chiara Indian et al.* Protein Science **10** (2001) 108.