Journal of Food and Nutrition Research, 2016, Vol. 4, No. 9, 566-570
Available online at http://pubs.sciepub.com/jfnr/4/9/2

DOI:10.12691/jfnr-4-9-2

Effect of Ultrasound on the Hydrolytic Reaction of AS1.398 Neutral Protease

HU Ai-jun*, CAO Yuan-yuan, QU Ying, SONG Fei-ying, ZHENG Jie

Key Laboratory of Food Nutrition and Safety, Ministry of Education, Tianjin Food Safety & Low Carbon Manufacturing Collaborative Innovation Center, Institute for New Rural Development, Tianjin University of Science & Technology, Tianjin 300457, China
*Corresponding author: hajpapers@163.com

Abstract This study investigated the effect of ultrasonic irradiation on the hydrolytic reaction of AS1.398 neutral protease. Our results showed that the light absorbance increased dramatically under the combination of the three conditions, ultrasonic radiation, increased amount of enzyme and longer incubation period. This increase was also observed when enzyme amount and reaction duration kept constant, with ultrasonic radiation as the only variable. In addition, the changes in light absorbance followed a parabolic curve under conditions of increasing pH and temperature, reaching the peak at an optimal pH and temperature and then decreasing sharply. We concluded that ultrasonic irradiation could accelerate the velocity of the enzymatic reaction, however, did not alter the optimal pH and temperature of the reaction. With no ultrasonic irradiation, the enzyme kinetic equation was v=59.88[S]/(21.49+[S]). Ultrasonic irradiation increased the velocity constant, but decreased the Michaelis constant (Km), the equation became v=63.69[S]/(18.60+[S]) with ultrasound. In summary, ultrasound-assisted enzymatic activity of neutral protease led to higher hydrolysis.

Keywords: *ultrasonic irradiation, AS1.398 neutral protease, enzymatic hydrolysis*

Cite This Article: HU Ai-jun, CAO Yuan-yuan, QU Ying, SONG Fei-ying, and ZHENG Jie, "Effect of Ultrasound on the Hydrolytic Reaction of AS1.398 Neutral Protease." *Journal of Food and Nutrition Research*, vol. 4, no. 9 (2016): 566-570. doi: 10.12691/jfnr-4-9-2.

1. Introduction

Ultrasound is a kind of mechanic wave in elastic medium. As a form of energy, it can produce three effects on the medium: thermal effect, mechanic effect and cavitation effect [1]. Studies on application of ultrasonic techniques in enzyme engineering have become popular in recent years.

YU Shu-juan, et al. [2] studied the effect of ultrasonic radiation on enzymatic activity with and without substrate. Their results indicated that ultrasound modified the conformation of enzyme so as to change its activity. In addition, ultrasound accelerated the molecular moving of both solvent and product, changed the concentration difference and enhanced mass transfer coefficient. XIAO Yong-mei, et al. [3] researched the enzymatic synthesis of glucose esters under ultrasound, discovered that higher ultrasonic power led to the faster acceleration of reaction, and ultrasound did not change the properties and selectivity of the enzyme in the transesterification reaction. Yoshio's study [4] indicated the enzymatic activity was not changed with 15 hours' continuous treating of 20kHz, 15W ultrasound, and suggested that mild ultrasound was applicable in enzyme production. The reaearch of HUANG Zhuo-lie, et al. [5] showed that typsin activity increased 45.4% with ultrasonic treatment 3 min (15 kHz, 50W). Luo Denglin's [6] study found that ultrasound obviously improved the activity of inulinase produced by *Aspergillus niger*. In Carlos Magno R. Ribeiro [7], et al.'s article, the enzymatic hydrolysis of ethyl-3-hydroxy-3-phenylpropanoate using ultrasound was studied. The application of ultrasound led to an appreciative decrease in the reaction time of enzymatic hydrolysis without a significant change in the yield or enantiomeric excess of reaction products, when compared with the magnetic stirrer used. Azita Soltani, et al. [8] studied ultrasonic effect on enzymatic fibrinolysis, found that ultrasound could accelerate enzymatic fibrinolysis with adjunctive plasminogen activators. Additionally, ultrasound was known for interaction between biological substances on molecular level in sonodynamic therapy and sonochemistry. Therefore, they investigated the ultrasonic effect's possibilities to the biological activity of plasminogen activators that used in thrombolysis treatment. However, Antonio Vercetn [9] studied the inactivation of phospholipase A_2, α-chymotrypsin, trypsin and porcine pancreatic lipase by heat, and manothermosonication (MTS), and the simultaneous application of heat and ultrasound under the moderate pressure, in different treatment media. For different enzymes, their sensitivity to MTS varied greatly. Whereas phospholipase A_2 was almost insensitive to MTS treatments. MTS inactivation of α-chymotrypsin and porcine lipase was much faster than heat inactivation, and typsin's thermal inactivation was different at low and high temperature. At low temperatures, it did not follow first order kinetics, contrary to what happened at high temperatures. MTS accelerated trypsin inactivation only at

low temperatures. MTS changed also the inactivation order of trypsin (at low temperatures), α-chymotrypsin and porcine lipase; whereas heat inactivation of these enzymes did not follow first order kinetics, MTS inactivation fitted this well [9].

AS1.398 neutral protease is an extra cellular protease which derives from *Bacillus subtilis*. It hydrolyzes protein directly into peptides and parts of free amino acids [10]. It is widely used in leather desquamating, cinefilm recycling, beer clarification, and protein hydrolysis of animals and plants [11].

Till now, no researches about effect of ultrasound on AS1.398 protease's properties were found. In this article, it was investigated in order to provide some practical and theoretical background to the application of ultrasound and the enzyme in food science and technology.

2. Experiments

2.1. Effect of Enzyme Amount on the Hydrolytic Reaction with and without Ultrasound

1g enzyme powder (its activity was 50000 U/g) was diluted to 200mL with buffer solution, then 2Ml, 3mL, 4mL, 5mL, 6mL and 7mL of above solution was taken and diluted to 100mL separately, thus enzyme solutions with the activity of 5U/mL, 7.5U/mL, 10U/mL, 12.5U/mL, 15U/mL were prepared.

For the hydrolytic reaction without ultrasound, the enzyme and substrate reacted 10 min under the following conditions: casein concentration 0.5%, pH 7.5 and reaction temperature 50°C. And for the reaction with ultrasound, the conditions were the same except the application of 108W ultrasound. When reaction time reached, the reaction solution was centrifugated, and its supernatant was taken to determine the light absorbance at 275nm [12].

2.2. Effect of pH on the Hydrolytic Reaction with and without Ultrasound

1g enzyme powder was diluted to 4000 mL with different pH phosphoric acid buffer solution, thus different enzyme solutions (12.5 U/mL) with pH6, pH 6.5, pH 7, pH 7.5, pH 8 and pH 8.5 were prepared separately. The enzyme and substrate reacted at above different pH for 10 min with the conditions of enzyme activity 12.5 U/mL, casein concentration 0.5%, and reaction temperature 50°C. Additionally, 108W ultrasound was used to the ultrasound-assisted enzymic reaction. When reaction ended, the solution was centrifugated, and its supernatant was taken to determine the light absorbance at 275 nm.

2.3. Effect of Temperature on the Hydrolytic Reaction with and without Ultrasound

Enzyme and substrate reacted at different temperature 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, and with the protease of 12.5U/mL and pH 7.5. The reaction conditions were the same as those mentioned in 2.1. When reaction ended, we centrifugated the solution, and took above clear liquid to determine its light absorbance at 275 nm.

2.4. Effect of Reaction Time on the Hydrolytic Reaction with and without Ultrasound

Enzyme and substrate reacted with 0W and 108W ultrasound, the other conditions for both reactions were casein concentration 0.5%, pH 7.5, reaction temperature 50°C, and the enzyme activity 12.5 U/mL. The reaction time was 5min, 10min, 15min, 20 min, 25 min and 30 min separately.When reaction ended, we centrifugated the solution, and took above clear liquid to determine its light absorbance at 275 nm.

2.5. Plot of Tyrosine Standard Curve

100 mg dry tyrosine was weighed with an accuracy of 0.2 mg and dissolved to 100 mL by 1 mol/L hydrochloric acid, thus 1 mg/mL standard tyrosine solution was prepared. Then above 10 mL solution was taken and quantified to 100 mL with 0.1 mol/L hydrochloric acid. 100 μg/mL tyrosine standard solution was prepared. Separately, the solution of 0, 1, 2, 3, 4 and 5 mL was taken, then 10, 9, 8, 7, 6 and 5 mL distilled water was added to prepare different tyrosine solutions with 0, 10, 20, 30, 40, 50 μg/mL. After determining the absorbance of above solution, the standard curve was plotted as follow (Figure 1).

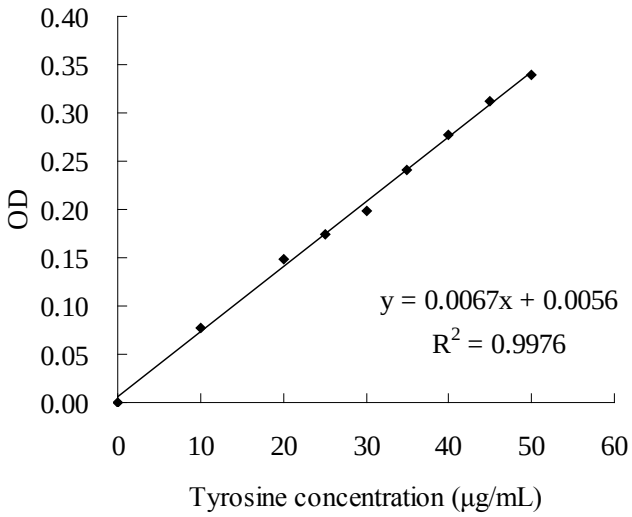

Figure 1. Tyrosine standard curve

2.6. Enzyme Reaction Kinetics with and without Ultrasound

Taking casein as a substrate, AS1.398 neutral protease with and without ultrasound was used to hydrolyze it at pH 7.5 and 50°C. The substrate concentration was seperately 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL and 8mg/mL, and reaction time was 0 min, 5 min, 10 min, 15 min, 20 min, 25 min and 30 min respectively. The hydrolysate was taken to determine its light absorbance at each substrate concentration and reaction time mentioned above. By the relationship of velocity reciprocal (1/υ) and substrate reciprocal (1/S), the Michaelis constant (K_m) and maximum velocity (v_{max}) were gotten, thus the enzyme reaction kinetics with and without ultrasound were also known.

3. Results and Discussion

3.1. Effect of Enzyme Amount on the hydrolytic Reaction with and without Ultrasound

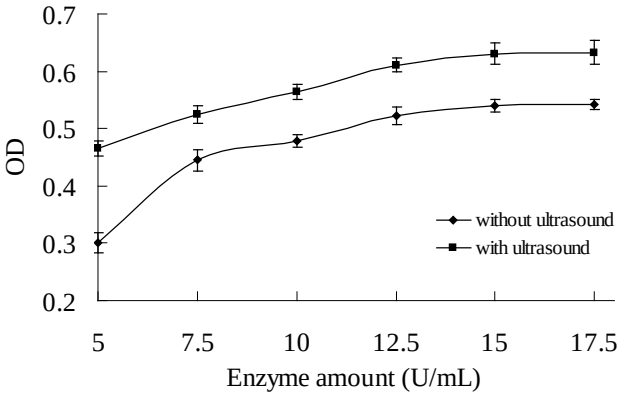

Figure 2. Effect of enzyme amount on enzymatic reaction with and without ultrasound

Figure 2 indicated that the hydrolysate's light absorbance (OD) increased with increasing enzyme amount for both reactions with and without ultrasound. At the same enzyme amount, the OD with ultrasound was obviously higher than that without ultrasound. As known to us that ultrasound-assisted enzymatic hydrolysis produced much more end product. The most possible reason is that ultrasonic irradiation may have three physical effects: thermal effect, mechanical effect and cavitation effect in view of physics, owing to these effects, the molecules of substrate and product move faster, additionally, the enzyme activity maybe improved because of its possible conformational modification by ultrasound [13], so that the hydrolysis was improved and the OD increased.

3.2. Effect of pH on the Hydrolytic Reaction with and without Ultrasound

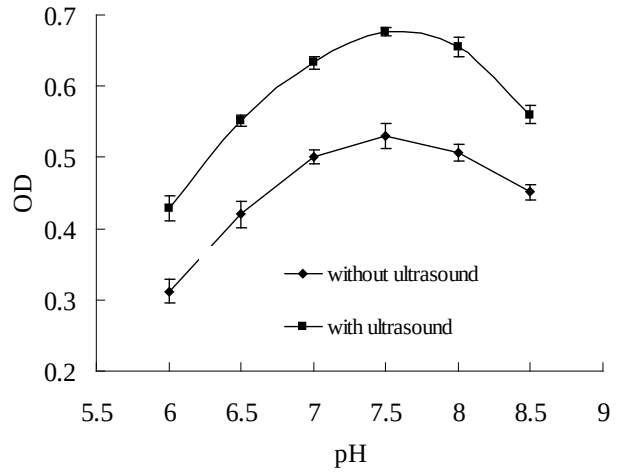

Figure 3. Relationship of OD and pH

Figure 3 showed whether ultrasound was used or not, the OD first went up then went down, and both OD curves appeared like parabolic shape with the change of reaction solution pH. When the solution pH changed from 6.0 to 8.5, the OD with ultrasound was higher than that without ultrasound at the same pH, which indicated that ultrasound improved the velocity of enzymatic reaction. Figure 3 also indicated that ultrasound did not change the optimal pH of enzyme reaction.

pH can influence not only enzyme configuration, but also the ion state of enzyme, substrate and enzyme-substrate complex. And it can also effect ultrasonic cavitation [1]. There may be why the enzymatic activity or reaction velocity is affected.

3.3. Effect of Temperature on the Hydrolytic Reaction with and without Ultrasound

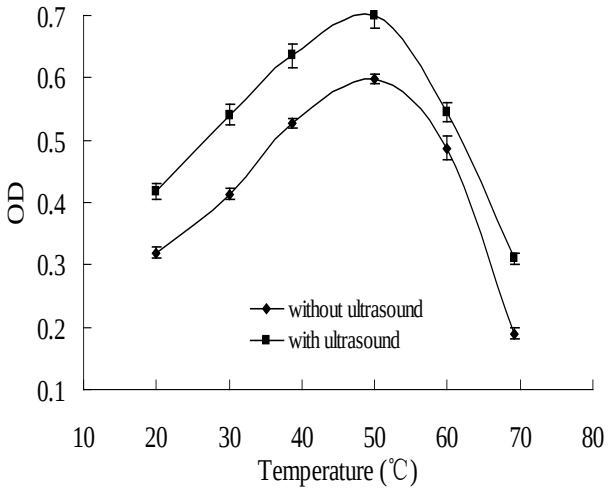

Figure 4. Relationship of OD and temperature

As seen in Figure 4, for two curves with and without ultrasound, the OD firstly increased and then reduced with reaction temperature increasing. When the temperature was 50°C, it reached the maximum for the reactions with and without ultrasound. Ultrasound made the OD obviously higher than that without ultrasound. With ultrasound, the OD showed an increase of 17.05% and 20.45% at 40°Cand 50°C separately than that without ultrasound. It was also seen in Figure 4 that ultrasound did not change the optimal temperature of enzyme reaction.

It is known that temperature influence not only enzyme activity, but also ultrasonic cavitation effect and mass transfer coefficient, consequently influence the enzymatic hydrolysis degree [1]. Generally speaking, every enzyme has its optimal reaction temperature, at which, enzyme reaction velocity reaches the maximum, and hydrolysis degree gets to the highest. On the other hand, the formation of the ultrasonic cavitation and its intensity closely depend on temperature. A moderate temperature can enhance ultrasonic effects, and ultrasonic cavitation has effects on enzyme reaction [13], so reaction temperature influences enzymatic reaction.

3.4. Effect of Reaction Time on the Hydrolytic Reaction with and without Ultrasound

Figure 5 indicated that the OD increased with the extension of reaction time. With ultrasound it was bigger than that without ultrasound at the same reaction time. It clearly stated ultrasound accelerated enzymatic reaction, so that the OD improved.

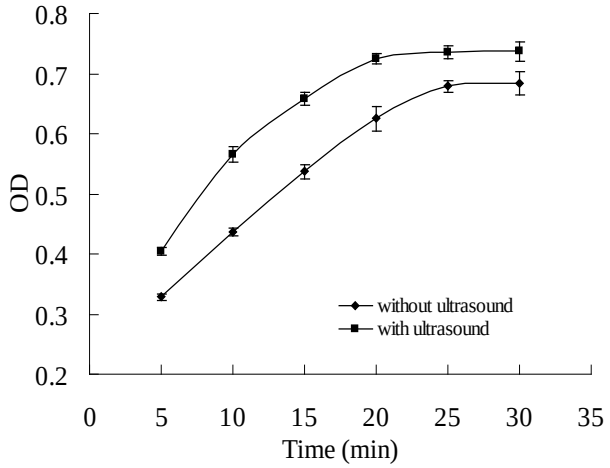

Figure 5. Relationship of OD and reaction time

3.5. Kinetic Equation of Neutral Protease Reaction without Ultrasound

According to tyrosine standard curve (Figure 1), the process curves of neutral protease reaction were gotten in form of tyrosine concentration (see Figure 6).

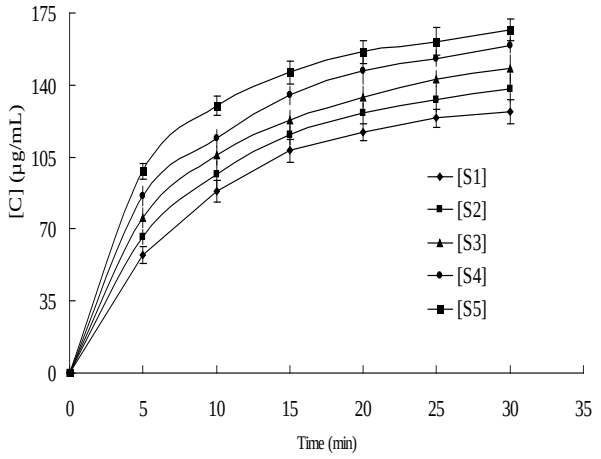

Figure 6. Relationship of tyrosine concentration, substrate concentration and reaction time (without ultrasound) ([S1]:4 mg/mL, [S2]:5mg/mL, [S3]:6mg/mL, [S4]:7mg/mL, [S5]:8mg/mL)

On the base of Figure 6, the relationship of velocity reciprocal (1/v) and substrate reciprocal (1/[S]) was plotted in Figure 7.

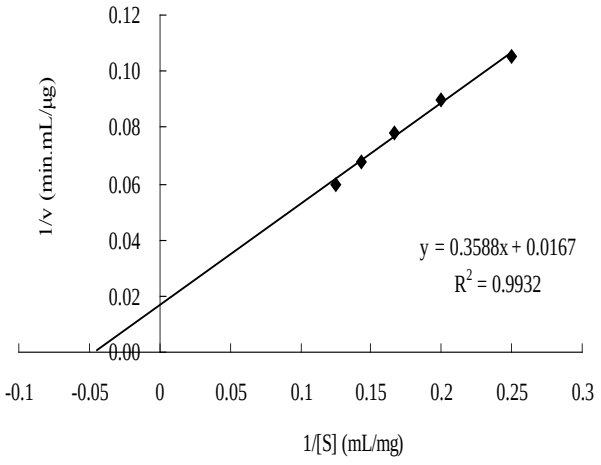

Figure 7. Relationship of 1/v and 1/[S] (without ultrasound)

According to Figure 7, it was shown that the straight line intercept was 0.0167, so $1/v_{max}$ = 0.0167 min.mL/μg, v_{max}=59.88μg/mL.min; and its slope coefficient was 0.3588, so K_m/v_{max}=0.3588, K_m=0.3588×v_{max}=21.49mg/mL. Thus, the kinetic equation of AS1.398 neutral protease reaction without ultrasound was v=59.88[S]/(21.49+[S]).

3.6. Reaction Kinetic Equation of Neutral Protease with Ultrasound

The process curves of neutral protease reaction with ultrasound were expressed in form of tyrosine concentrate in Figure 8.

According to tyrosine standard curve (Figure 1) and Figure 9, the process curves of neutral protease reaction with ultrasound were gotten in form of tyrosine concentration (see Figure 8).

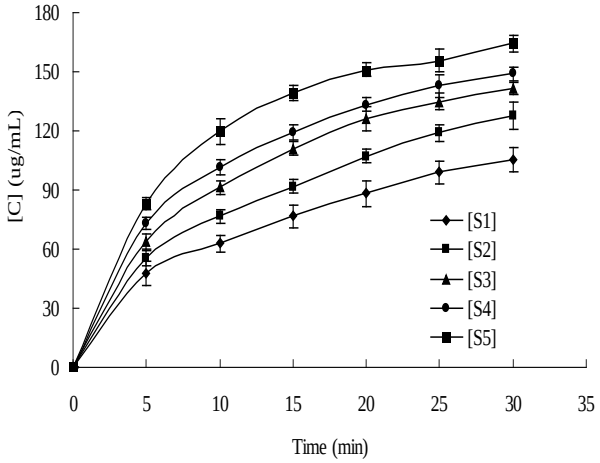

Figure 8. Relationship of tyrosine concentration, substrate concentration and reaction time (with ultrasound) ([S1]:4 mg/mL, [S2]:5mg/mL, [S3]:6mg/mL, [S4]:7mg/mL, [S5]:8mg/mL)

On the base of Figure 8, the relationship of velocity reciprocal (1/v) and substrate reciprocal (1/[S]) was plotted in Figure 9.

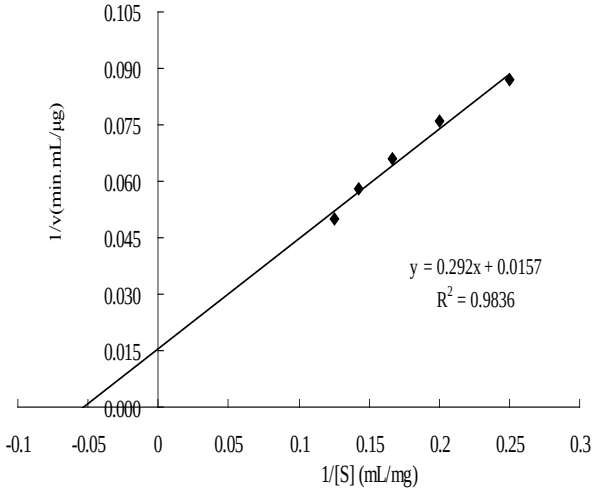

Figure 9. Relationship of 1/v and 1/[S] (with ultrasound)

Figure 9 showed that the straight line intercept was 0.0157, so $1/v_{max}$ =0.0157min.mL/μg, v_{max} =63.69μg/mL.min; and its slope coefficient was 0.292, so K_m/v_{max}=0.292, K_m=0.292×v_{max} =18.60mg/mL. Thus, the kinetic equation

of AS1.398 neutral protease reaction with ultrasound was v=63.69[S]/(18.60+[S]).

4. Conclusions

(1) For both hydrolytic reactions with and without ultrasound, the hydrolysate's light absorbance increased with raising enzyme amount or extending reaction time. Compared with that without ultrasound, ultrasound-assisted enzymatic hydrolysis produced much more end product at the same conditions else.

(2) With increasing of pH and temperature, for both reactions with and without ultrasound, the hydrolysate's light absorbance firstly increased then decreased. Ultrasound improved the velocity of enzymatic reaction, however, it did not change the optimal pH and temperature of enzyme reaction.

(3) Without ultrasound, the kinetic equation of neutral protease reaction was v=59.88[S]/(21.49+[S]); and with ultrasound, it was v=63.69[S]/(18.60+[S]). Compared with no ultrasound, for the enzymatic reaction with ultrasound, its v_{max} became bigger, and K_m was smaller.

Acknowledgement

The authors acknowledge the financial support from the Industry-university-institute Cooperation Project of Special Fund for Development of Small and Medium-sized Technology-based Firms of Dongli District of Tianjin (Project title: Development and Application on Key Technology of Compound Surimi Products Processing), the China Postdoctoral Science Foundation funded project (Grant No.: 2013M540211), and the Project of Science and Technology Correspondent of Tianjin City (Project title: Development on Key Processing Technology and Products of Loach Snack Food). The authors are also grateful for the help and cooperation from Tianjin Hongteng Aquatic Science and Technology Development Co., Ltd.

References

[1] HU Ai-jun, ZHENG Jie. Comparison between soybean peptides production by enzymatic hydrolysis with and without ultrasound. Food Science and Technology, 2005, (3):27-30.

[2] YU Shu-juan. A study on enzymatic extraction of ganoderma under ultrasonic field, Ph.D Dissertation submitted to South China University of Technology, Guangzhou, 1996.

[3] XIAO Yong-mei, QI Wu, YING Cai, et al.. Ultrasound-accelerated enzymatic synthesis of sugar esters in nonaqueous solvents. *Carbohydrate Research*, 2005, 340(13): 2097-2103.

[4] Yoshio Tshori, Lsao Karube, Shuichi Suzuki. Continuous production of glucose oxidase with *aspergillus* sp under ultrasound waves. *Enzyme Microb. Technol*., 1982, (4):85-88.

[5] HUANG Zhuo-lie, CHEN Xiao-li, WU Guang-hong, et al.. Study on the effect of ultrasonic wave on the activity of trypsin. Chinese Journal of Biochemical Pharmaceutics. 2009, 30(4): 230-238.

[6] LUO Deng-lin, YUAN Hai-li, WANG Fei, et al. Effect of ultrasound on inulinase production fron aspergillus niger fermentation. Technical Acoustics, 2012, 05:502-505.

[7] Carlos Magno R. Ribeiro, Elisa N. Passaroto, Eugênia C. S. Brenelli. Ultrasound in enzymatic resolution of ethyl 3-hydroxy-3-phenylpropanoate. *Ttrahedron Letters*, 2001, 42(37):6477-6479.

[8] Azita Soltani, Charles Soliday. Effect of ultrasound on enzymatic activity of selected plasminogen activators. *Thrombosis Research*, 2007, 119(2):223-228.

[9] Antonio Vercet, Justino Burgos, Simon Crelier, et al.. Inactivation of proteases and lipases by ultrasound. *Innovative Food Science & Emerging Technologies*, 2001, 2(2):139-150.

[10] ZHANG Shu-zheng. Industry of Enzyme Products. Publishing House of Science.1984, 430.

[11] TANG Zi-jin, XIAO Jun-fang. Study on the character of immobilized AS1.398 neutral protease. *Food Science*, 1995, 16 (6): 6.

[12] HUANG Chun-xi., LIAO Chun-feng, HUANG Yi-hui, et al. Study on the determination of the activity of Papaya with an accurate and rapid method. Light Industry Science and Technology, 2012, 16:48.

[13] HU Ai-jun, QIU Tai-qiu. Application of ultrasound technique in food industry. Technical Acoustics, 2002, 04:192-194+199.