CHARACTERIZATION OF PROTEASE FROM *ASPERGILLUS ORYZAE* SURFACE CULTURE AND APPLICATION IN FISH SAUCE PROCESSING

Le Van Viet Man, Tran Thi Anh Tuyet
Department of Food Technology, University of Technology, VNU-HCM
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ABSTRACT: *Some kinetic parameters of the purified protease from Aspergillus oryzae surface culture were determined: K_m=634.5mg/L and V_{max}=13.98mg/L.min. Ions Na^+, Zn^{2+} and Fe^{3+} had an inhibitor effect to the enzyme activity. On the contrary, ion Mg^{2+} had an activator effect. The influence of ion Ca^{2+} to the protease activity was more complicated. With low concentration (0.005M), this ion activated the protease; but with higher concentration (0.01M), it inhibited the enzyme activity. The purified protease was applied in fish sauce processing for proteolysis acceleration. High salt content (25%) in the fish-salt mixture decreased obviously the enzyme activity. Addition of salt and fungal protease to the fish sample with several times accelerated significantly fish proteolysis.*

1. INTRODUCTION

Fish sauce has been a traditional product in Vietnam. Production time of fish sauce varies from 6 to 12 months. Application of microbial protease in fish sauce processing permits to decrease the production time and increase the equipment productivity (Steinkraus K. H., 1983).

Salt tolereance is an important charactersitics of commercial protease used in fish sauce processing. Different commercial enzymes with high protease activity cannot be used in fish sauce processing because the enzymes are non salt-tolerant. These proteases are significantly inhibited by a salt concentration of 10-20%.

Aspergillus oryzae can produce salt-tolerant protease. In some Asian countries, this fungi has widely been applied in the production of different traditional fermented foods with high salt content such as soy bean sauce.[3]

This study focussed on the characterization of protease from *Aspergillus oryzae* surface culture. Firstly, the influence of different ions to the enzyme activity was examined. Then the kinetic parameters K_m and V_{max} of the obtained protease were determined. Finally, the enzyme was applied in fish sauce processing for decrease of proteolysis time.

2. MATERIALS AND METHODS

2.1. Materials

Micro-organism: A strain of *Aspergillus oryzae* from the Culture collection of Food Microbiology Laboratory, Department of Food Technogy, Ho Chi Minh City University of Technology was used. Rice bran (Starch – 15.3%, crude protein – 14.8%) and soy meal (Crude protein – 38.5%) were used as medium substrates for protease biosynthesis by *A. oryzae.*

Anchovy fish (Crude protein – 15.8%) was used in fish sauce fermentation. Chemicals used in this study were supplied by Sigma, Merck and a Chinese company.

2.2. Protease production from *Aspergillus oryzae* surface culture

Medium composition for protease biosynthesis: Medium contained rice bran – 83%, soy meal – 12.7% and rice husk - 4.3%. Initial moisture of the medium was 50%. Medium was sterilized at 120°C for 20 minutes..[2]

Conditions for protease biosynthesis: stainless steel cuvets (200*400*5mm) were used for solid state fermentation. Medium in cuvet was inoculated by a spore suspension of *A. oryzae.*

Medium height in cuvet was not exceed 3mm. Solid state fermentation was carried out at 30°C for 48 hours. [2]

Protease extraction: The optimal conditions for protease extraction from *A. oryzae* surface culture were as follows: solvent – phosphate buffer (pH = 6.5), ratio of surface culture and solvent – 1/8 (w/w), temperature – 20°C, time – 35 minutes, agitation speed – 200rpm. After the enzyme extraction, the sample was centrifuged at 5°C for 20 minutes for eliminating the biomass and non solubles subtances. [2]

Protease purification: Enzyme was precipitated by organic solvent. The ratio of crude enzyme extract and ethanol 95% was 65/35 (v/v). The protease recovery yield was 65.45% and the purified degree was 3.03. This purified protease was used for determination of K_m, V_{max} and applied in fish sauce processing.

2.3. Determination of kinetic parameters K_m and V_{max}

Serum bovine albumine was used as a substrate. The substrate concentration varied from 100 – 5000mg/L. The enzyme concentration was fixed: 0.1 units per 1g albumine. The proteolysis was carried out at 50°C, pH = 7.0.

Product of proteolysis was evaluated by free amino nitrogent content. From the experimental data, the kinetic equation of free amino nitrogen accumulation was determined by Excel software. K_m and V_{max} were determined by Lineweaver – Burk method .[1]

2.4. Application of fungal protease in fish sauce processing

Each sample contained 100g anchovy fish. The purified protease from *A. oryzae* was added with different contents and at various moments of the fish sauce fermetation according to each experiment.

2.5. Analytical methods

- Protease activity was determined by modified Anson method.
- Enzyme protein was quantified by Lowry method, using Serum Bovine Albumine as a standard.
- Crude protein contents in fish, rice bran and soy meal were determined by Kjeldahl method.
- Amino nitrogen was quantified by spectrophotometric method using ninhydrin reagent.

3. RESULTS AND DISCUSSION

Our prelimenary study shows that the optimal pH and temperature of the protease produced by *Aspergillus oryzae* surface culture are 7.0 and 50°C respectively. In addition, the enzyme is fairly salt-tolerant. In the sample with NaCl 50g/L and 100g/L, the enzyme activity remained 70% and 50% in comparison with the control sample.[2]

3.1. Influence of different ions to the protease activity

Figures 1 and 2 show the influence of some popular ions in food processing to the relative activity of protease from *A. oryzae* surface culture. Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+} in sulfate and chloride salts 0.005M were used in this study. It was noted that in both cases, ion Mg^{2+} activated the enzyme. On the contrary, ions Fe^{3+}, Zn^{2+} inhibited the protease acivity. In the presence of sulfate ion, the enzyme was stronger inhibited by Fe^{3+}, Zn^{2+} than in the presence of chloride ion. With regard to the ion Ca^{2+}, its influence to the enzyme catalysis was more complicated. In the presence of chloride ion, ion Ca^{2+} was an enzyme activator. But in the presence of sulfate ion, the content of ion Ca^{2+} 0.005M did not affect to the enzyme activity.

The influence of the 4 ions above with higher concentration (0.01M) to the protease activity is visualized in figures 3 and 4. Ion Mg^{2+} increased the catalytic activity of the enzyme.

However, the higher the concentration of ion Mg^{2+}, the lower the activation effect. Three ions Fe^{3+}, Zn^{2+}, Ca^{2+} in both sulfate and chloride salts decreased the enzyme activity. The highest inhibitor effect belonged to ion Fe^{3+}.

It can be noted that the higher the concentrations of the ions, the higher the inhibition effects to the protease activity. For explaining this phenomenon, a study of the enzyme structure is essential. In food processing, different ions are presented in raw materials and water. Study of ion modulators permits to regulate efficiently the enzymatic reactions in industrial scale.

3.2. Determination of kinetic parameters K_m and V_{max}

The kinetic parameters K_m and V_{max} were calculated from the 1/S against 1/V plot by Lineweaver – Burk method, using initial reaction rates for different concentrations of substrates (Figure 5). They were found to be 634.45 mg/L and 13.99 mg/L.min respectively. The same kinetic parameters were also observed for other commercial prteases.[1]

Figure 6 presents the relation between substrate concentration and reaction rate. The protease from *A. oryzae* surface culture followed a typical type of Michaelis – Menten kinetics. It shows that if the protein concentration in the proteolytic sample was about 5 – 10 g/L, the reaction rate reached maximum.

3.3. Application of fungal protease in fish sauce processing

In fish sauce fermentation, proteolysis is carried out by 2 enzyme sources: protease in the digestion systems of fish and the protease produced by the micro-organisms existed on fish. Addition of commercial proteases, for example – protease from Bacillus subtilis - could decrease the proteolytic time.

Nevertheless, high ratio of salt/fish (25% w/w) in fish sauce fermentation prolonged the proteolysis. In this experiment, procedure of salt addition in fish sauce fermentation was studied. Three samples S1, S2 and S3 were examined. The experimental plan is given in table 1. The final ratio of salt/fish in the three sample were the same – 25%. The purified protease from *A. oryzae* was added: 0.075 units/g fish protein.

Table 1. Procedure of salt addition - Experimental plan

Time (days)	Sample 1 (S1)	Sample 2 (S2)	Sample 3 (S3)
	Salt addition – Ratio salt/fish (w/w)		
0	5	10	15
1	5	10	10
2	5	5	
3	5		
4	5		

Figure 7 illustrates the obtained results. After 1 day, the ratio of salt/fish in sample S3 reached maximum (25%). The proteolysis seemed to be stopped after the first 2 days. Prolongation of fermentation time (more than 2 days) did not increase free amino nitrogen content in the sample. It can be confirmed that high concentration of salt inhibited the protease acitivity. The maximal free amino nitrogen content in sample S3 was about 5.18g/L.

In sample S2, after 2 days, the ratio of salt/fish reached maximum (25%). The proteolysis slowed down after the first 3 days. The maximal free amino nitrogen content in sample S2 was 6.32g/L - 22% higher than that in sample S3.

Regard to sample S1, after the first 4 days, salt addition was completed. The proteolysis continued up to the 5th day. The free amino nitrogen concentration was the highest (11.64 g/L). It was 125% and 84% higher than those in samples S3 and S2 respectively. In summary, procedure of salt addition in fish sauce processing is very important. The procedure in the sample S1 seems to be satisfied for both purposes: inhibiting the contaminated and pathogenic micro-organisms in fish and maintaining the protease acitivity during the fermentation.

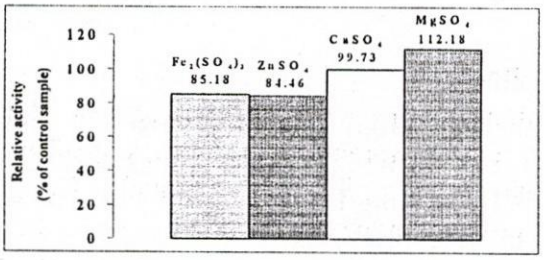

Fig. 1: Influence of Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+} sulfate salt 0.005M) to the protease activity

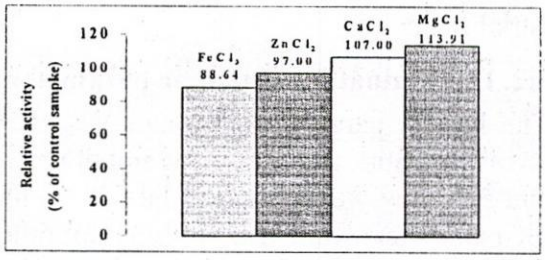

Fig. 2: Influence of Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+} (chloride salt 0.005M) to the protease activity

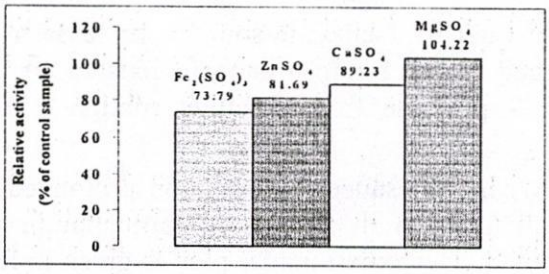

Fig. 3: Influence of Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+} (sulfate salt 0.01M) to the protease activity

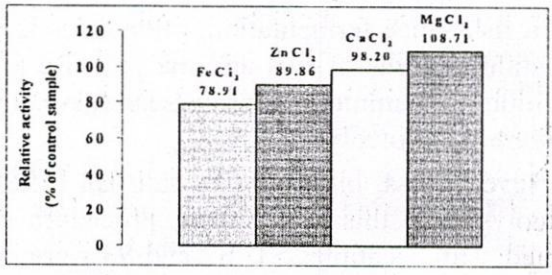

Fig. 4: Influence of Fe^{3+}, Zn^{2+}, Ca^{2+} and Mg^{2+} (chloride salt 0.01M) to the protease activity

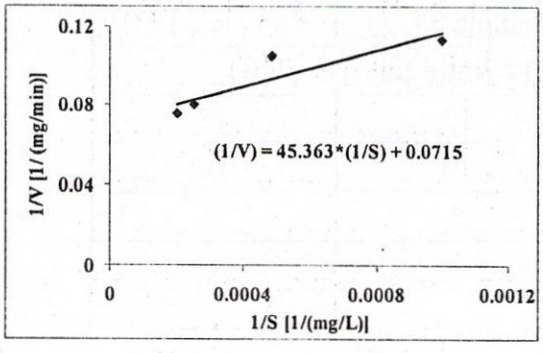

Fig.5: Lineweaver - Burk plot of experimental data for protease from *A. oryzae* surface culture: K_m = 634.45 mg/L; V_{max}=13.99 mg/L.min)

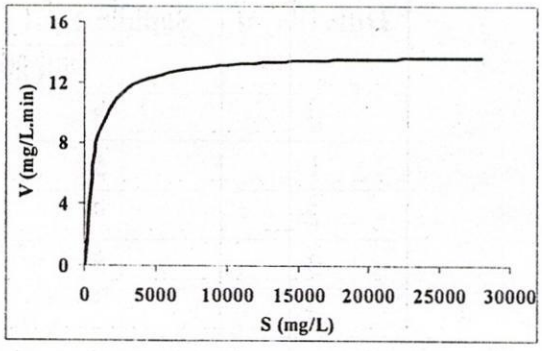

Fig.6: Relation between substrate concentration and reaction rate (protease concentration: 5 units/ g protein

In the next experiment, various contents of exogenous protease was added to the fish sample at different moments of the fermentation.The experimental plan is presented in table 2.

The procedure of salt addition was carried out as in the sample S1. The obtained results are given is fugure 8.

Table 2. Influence of exogenous protease content to fish proteolysis - Experimental plan

Time (days)	Sample 4 (S4)	Sample 5 (S5)	Sample 6 (S6)	Sample 7 (S7)	Sample 8 (S8)
	Exogenous protease addition – Units/g fish protein				
0	0.004	0.008	0.012	0.016	0.02
2	0.004	0.008	0.012	0.016	0.02
4	0.004	0.008	0.012	0.016	0.02
6	0.004	0.008	0.012	0.016	0.02
Total content of fungal protease	0.016	0.032	0.048	0.064	0.080

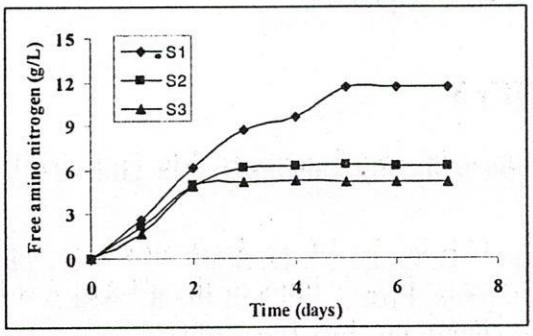

Fig. 7: Influence of salt addition procedure to the evolution of free amino nitrogen content during fish proteolysis

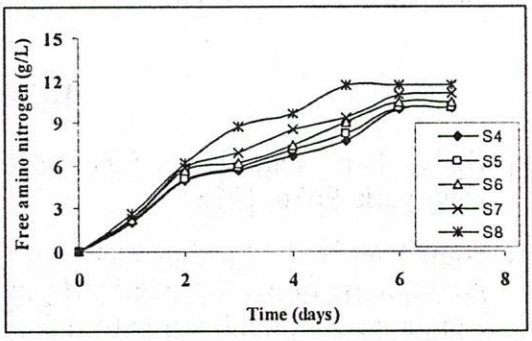

Fig. 8: Influence of protease content from *A. oryzae* to the evolution of free amino nitrogen concentration during fish proteolysis

Figure 8 shows that the higher the content of exogenous protease, the stronger the proteolysis and the higher the free amino nitrogen in the sample. However, the content of the commercial protease was limited because of economic reasons.

For the control sample without exogenous enzyme, after the first 7 days, the free amino nitrogen was about 4 g/L. In comparison with sample S8, it reached 11.69 g/L – 192% higher than that in the control sample. The protease from *A. oryzae* surface culture was therefore very suitable in fish sauce processing for proteolysis acceleration.

4. CONCLUSION

Some characteristics of the protease from *A. oryzae* surface culture were determined. The kinetic parameters were as follows: K_m = 634.45 mg/L, V_{max} = 13.99 mg/L.min. The ion Mg^{2+} in concentration of 0.005M and 0.01M activated enzyme. On the contrary, the ions Fe^{3+}, Zn^{2+} and Ca^{2+} in concentration of 0.01M decreased the enzyme activity.

This protease was fairly salt-tolerant. The application of this enzyme with a suitable procedure of salt addition in fish sauce processing accelerated fish proteolysis and increased the free amino nitrogen content. Our research continued on optimization of technological parameters of fish proteolysis for amelioration of free amino nitrogen content and flavour of fish sauce.

KHẢO SÁT MỘT SỐ TÍNH CHẤT CỦA CHẾ PHẨM PROTEASE TỪ CANH TRƯỜNG NUÔI CẤY BỀ MẶT *ASPERGILLUS ORYZAE* VÀ ỨNG DỤNG ENZYME TRONG SẢN XUẤT NƯỚC MẮM

Lê Văn Việt Mẫn, Trần Thị Ánh Tuyết
Bộ môn Công nghệ Thực phẩm, Trường Đại học Bách khoa, ĐHQG-HCM

TÓM TẮT: *Các thông số động học của chế phẩm protease từ canh trường Aspergillus oryzae đã được xác định: Km=634.5mg/L và Vmax=13.98mg/L.min. Kết quả thực nghiệm cho thấy ion Na^+, Zn^{2+} và Fe^{3+} đều là những tác nhân ức chế hoạt tính enzyme. Ngược lại, ion Mg^{2+} là tác nhân hoạt hóa. Sự ảnh hưởng của ion Ca^{2+} đến hoạt tính chế phẩm protease theo một quy luật phức tạp hơn. Ở nồng độ thấp (0.005M), ion Ca^{2+} làm tăng hoạt tính enzyme, nhưng ở nồng độ cao (0.01M), ion Ca^{2+} lại ức chế khả năng xúc tác của protease. Tiếp theo, chúng tôi thử ứng dụng chế phẩm protease trong sản xuất nước mắm nhằm rút ngắn thời gian thủy phân protein. Nồng độ cao của muối trong chượp (25% khối lượng) đã làm giảm hoạt tính xúc tác của chế phẩm. Việc áp dụng chế độ bổ sung muối và chế phẩm protease nhiều lần vào chượp sẽ làm rút ngắn đáng kể thời gian thủy phân protein cá trong chượp.*

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