J Food Sci Technol (May–June 2011) 48(3):280–284
DOI 10.1007/s13197-010-0128-8

ORIGINAL ARTICLE

The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil

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Revised: 2 March 2010 / Accepted: 29 September 2010 / Published online: 5 November 2010
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Abstract The technology of enzymatic extraction of fish oil has many advantages, such as moderate operating conditions, lower energy consumption and high efficiency. Moreover, it could reduce the breakage for the functional component of fish oil. In enzymatic extraction of oil, the investigation of the property of enzymatic reaction is propitious to improve the enzymolysis efficiency. In this study, the 1398 neutrase was used for hydrolyzing fish protein, then analyzed the impacts to the enzymolysis efficiency which were induced by the different initial substrate concentration and different initial enzyme concentration, the result showed that the higher substrate concentration generate inhibition to the enzyme. And then, the properties of enzymatic hydrolysis were studied by the Michaelis-Menten equation of substrate inhibition and the enzymatic hydrolysis kinetics equation which is derived by theory. By means of the verification to the enzymatic hydrolysis kinetics model, it could see that the model in line with the actual situation better at a lower degree of hydrolysis. Lastly, the critical enzyme concentration and critical substrate concentration of enzymatic reaction could be obtained by deducing the enzymatic hydrolysis kinetics model.

Keywords Fish oil · Degree of hydrolysis · Substrate inhibition · Michaelis-Menten equation · Enzymolysis efficiency

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Introduction

Fish oil is rich in EPA, DHA, fat-soluble vitamins and other nutrient ingredients (Wu 2004; Eslick et al. 2009). EPA and DHA have many functions, including anti-platelet aggregation, thrombosis delay, tumor inhibition, antirheumatic and eyesight protection (Carrero et al. 2007; Tisdale 2003; Chapkin et al. 2002; Bradley et al. 2001; Lu et al. 1996; Tidow-Kebritchi and Mobarhan 2001). With the study of fish oil progressing gradually, people's demands increasing year by year. The traditional technology of fish oil extraction including alkaline hydrolysis, expression method and direct drying method (Wu 2004), but the functional components of oil are always destroyed as a result of the adverse operating conditions in these operations. Enzymatic extraction of oil has aroused the concern of many scholars, because of the moderate operating conditions, low energy consumption and high efficiency (Posorske et al. 1984; Li et al. 1997; Fullbrook 1983), besides the by-product of hydrolysis contain all amino acids that are necessary to people (Yuan and Gao 2002), so it can be further processing and utilizing. Therefore enzymatic extraction of fish oil has been a hot spot in the present technology of oil extraction (Yang and Liu 2008; Hao et al. 2009). In order to obtain the higher extraction rate of fish oil with less enzymes, so it is necessary to investigate the properties of the enzymatic reaction.

In this study, the 1398 neutrase was used for hydrolyzing fish protein (Zhou 1994), then analyzed the impacts to the enzymolysis efficiency which were induced by the different initial substrate concentration and different initial enzyme concentration, the result showed enzymatic reaction velocity rising with substrate concentration

increasing when initial substrate concentration was lower, but the higher substrate concentration generated inhibition to enzyme. Then the property of enzymatic hydrolysis was studied by the Michaelis-Menten equation of substrate inhibition and the enzymatic hydrolysis kinetics equation which is derived by theory, and the value of parameters in the model of enzyme kinetics equation were obtained. By means of the verification to the enzymatic hydrolysis kinetics model, it could see that the model in line with the actual situation better at a lower degree of hydrolysis. Lastly, the critical enzyme concentration and critical substrate concentration of enzymatic reaction could be obtained by deducing the enzymatic hydrolysis kinetics model.

Materials and methods

Materials Fish was purchased from a local market in Hangzhou, China. After killing, the meat was broken up into fish sauce by high-speed organization broken machine. 1398 neutrase with the nominal activity of 1×10^5U/g was obtained from WUXI enzyme company, China. All other reagents were of the highest grade available commercially available grade.

Measurement of degree of hydrolysis (DH) DH is defined as the percentage of free amino groups cleaved from proteins, which was calculated from the ratio of α-amino nitrogen to total nitrogen. In this study, the degree of hydrolysis of protein was determinated by pH-stat method which used by Alder-Nissen. (1986).

Influence to enzyme reaction caused by different substrate concentration In enzymatic reaction, the effec caused by substrate concentration is significant, excessive amount of water will not propitious to the reaction and the relatively smaller quantity of substrate also affect titration results. So, according to single factor experiments (Qian et al. 2008), a series of substrates with different concentration (18.0 to 45.0 mg/mL) were prepared, then each group was added same amounts of enzyme, and other reaction conditions were fixed. The experiment was prepared in triplicates for each value. The degree of hydrolysis of protein was determined at different enzymolysis time. Thereby analyzed the influence to enzyme reaction which caused by different substrate concentration.

Influence to enzyme reaction caused by different enzyme concentration A series of substrates with same concentration were prepared, then each group was added different amounts of enzyme (0.8 to 1.4 mg/mL), and other reaction conditions were fixed. The experiment was prepared in triplicates for each value. The degree of hydrolysis of protein was determined at different enzymolysis time. Thereby analyzed the influence to enzyme reaction which caused by different enzyme concentration.

Determination of Michaelis constant The test of single factor of enzymolysis condition of fish showed that a higher substrate concentration generated inhibition to the enzyme (Qian et al. 2008).So, the reaction process as follows:

$$E + S \overset{k_1}{\longleftrightarrow} ES \tag{1}$$

$$ES + H_2O \xrightarrow{k_2} E + P \tag{2}$$

$$ES + S \overset{k_3}{\longleftrightarrow} ES_2 \tag{3}$$

Thereby the Michaelis-Menten equation of anti-competitive substrate inhibition was selected, and the equation as follows:

$$V = \frac{V_{\max}[S]}{K_s + [S]\left(1 + \frac{[S]}{K_s'}\right)} \tag{4}$$

Where K_2 is reaction rate constant of Eq. 2, the V_{max} is the maximum reaction velocity, K_S is the Michaelis constant and K_S' is the dissociation constant of ES_2. So if the initial reaction velocity under different substrate concentration is determined, then K_S, K_S' and V_{max} are all obtained by fit the data. The reaction velocity is proportional to the instillation velocity of lye according to the mensuration of DH, Alder-Nissen (1979), so analyse the relation between reaction time and alkali consumption, the initial reaction velocity under different substrate concentration will be obtained. Prepared a series of substrates with different concentration, and each group were added 0.2 g of neutrase, the enzymatic hydrolysis of fish was carried out with agitation (200 rpm) and at 45°C. Where the concentration of NaOH was 0.05 mol/L and the reaction volume was 200 mL. The experiment was prepared in triplicates for each value.

Determination of enzyme kinetic parameters The mechanism of enzymatic hydrolysis of protein is two substrate ordered reactions. The reaction Eq. 2 is the velocity-limiting step, and the inverse process could be neglected in this equation. In the enzymatic hydrolysis process, the enzymolysis velocity is proportional to the variation of

degree of hydrolysis, so it can be shown in following equation:

$$V = [S_0]\frac{d(DH)}{dt} = k_2[ES] \quad (5)$$

According to steady state theory, the relation of time and hydrolysis degree can be concluded and shown in following equation.

$$DH = \frac{1}{b}\ln(1 + abt) \quad (6)$$

Where

$$\frac{k_2[E]_0}{[S]_0} = a \quad (7)$$

$$\frac{k_d k_i}{k_2} = b \quad (8)$$

Where $[E]_0$ is the initial enzyme concentration and $[S]_0$ is the initial substrate concentration, K_d is the reaction rate constant of enzyme inactivation which is induced by the combination of complex (ES) and free enzymes. K_i is dissociation constant of complex (ES). So the parameter ("a" and "b") could be obtained through fitting the data of hydrolysis degree under different initial substrate concentration and initial enzyme concentration (Figs. 1 and 2).

Then a new enzymatic hydrolysis system was selected to test, with the initial substrate concentration is 18 mg/mL and the enzyme 0.4 g. The experiment was prepared in triplicates for each value. Recorded the degree of hydrolysis under different reaction time and compared to the theoretical value.

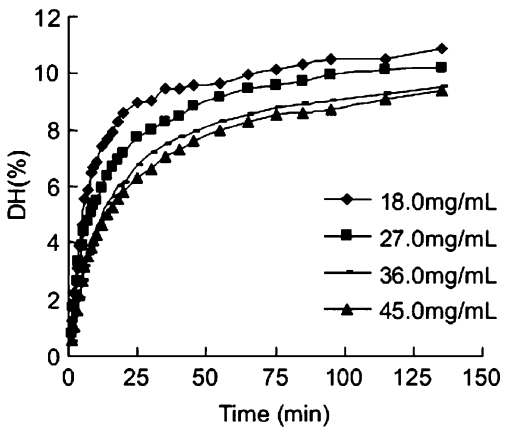

Fig. 1 DH under different concentration of substrate at different enzymatic hydrolysis time. The experiment was prepared in triplicates for each value (n=3)

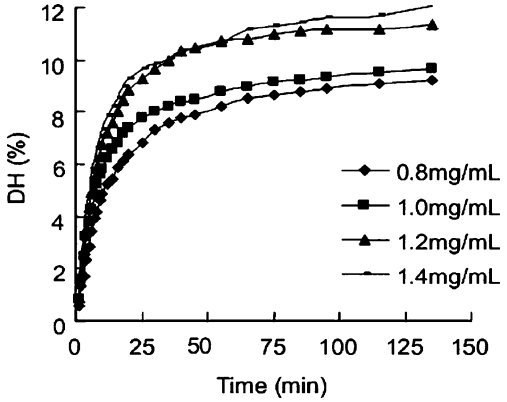

Fig. 2 DH of different enzyme concentration at different enzymatic hydrolysis time. The experiment was prepared in triplicates for each value (n=3)

Results and discussion

Effect to enzyme hydrolysis caused by different initial substrate concentration As shown in Fig. 1, at the same time, when the initial substrate concentration increased from 18.0 to 45.0 mg/mL, the degree of hydrolysis decreased. This result indicated that the higher substrate concentration generated inhibition to enzyme reaction. When the concentration of substrate was high fairly, the inhibitory effect will be strengthened. Therefore substrate not only can increase the reaction rate at lower concentration, but also can inhibit the activity of enzyme with higher concentration. So in the process of enzymatic hydrolysis of fish oil, an optimum substrate concentration should be selected to maintain a higher reaction rate as well as not generate inhibition to enzyme activity. In this way, the enzymolysis efficiency can be improved and the production cost can be reduced.

Effect to enzyme hydrolysis caused by different initial enzyme concentration As shown in Fig. 2, when the initial enzyme concentration increased from 0.8 to 1.4 mg/mL, the degree of hydrolysis increased obviously. The Fig. 2 also revealed that the enzymolysis rate declining as reaction time stretching, and the degree of hydrolysis tend to a different limits under a different enzyme concentration. And in practical applications, it is better to select a lower enzyme concentration according to the concrete conditions, in this way the degree of hydrolysis could be controlled easily and the catalyst cost could be reduced too.

Determination of Michaelis constant According to the relationship between the reaction time and alkali consumption, the enzymatic hydrolysis first velocity under different substrate concentration were obtained and depicted in Fig. 3. The Fig. 3 showed that at a lower substrate concentration (<10 mg/mL), enzymatic hydrolysis first

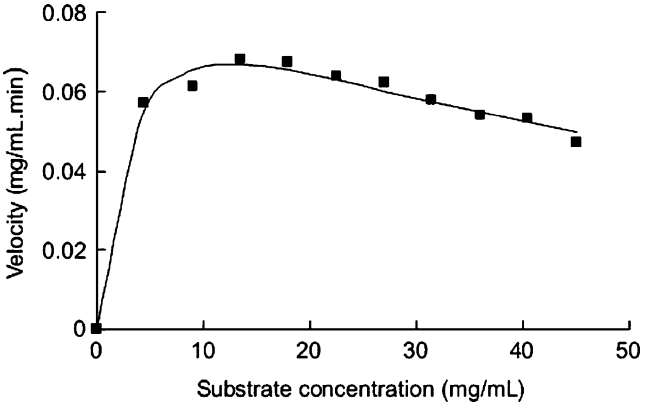

Fig. 3 Enzymatic hydrolysis first velocity under different concentration of substrate. The experiment was prepared in triplicates for each value (n=3)

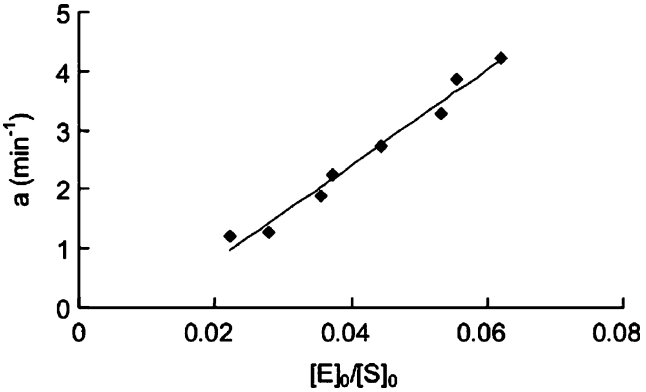

Fig. 4 Variation of "a" for different $[E]_0/[S]_0$ values

velocity increased with the concentration of substrate increasing. But when substrate concentration exceeded the value of 10 mg/mL, enzymatic hydrolysis first velocity decreased markedly with the concentration of substrate increasing. This phenomenon may be due to three reasons: High concentration of substrate may make molecule diffuse difficult in solution; If concentration of substrate is higher, the excess of substrate will combine to the complex (ES) and generate invalid complex (ES_2); The substrate with high concentration will contain lots of impurity which probably generate inhibition to enzyme reaction.

The data in Fig. 3 were fitted according to Eq. 4 by the DPS software (Home page, china), and the results as follows:

$$V = \frac{4.5386[S]}{3.6645 + [S]\left(1 + \frac{[S]}{42.7761}\right)} \tag{9}$$

The equation showed the value of V_{max}, K_s and K_s'. Many studies indicate that the value of K_s of neutrase and alkaline protease are less than 1%. Gel and casein were hydrolysed by nagarase at pH 8 and temperature (30°C), and the value of K_s were 0.5% and 0.1–0.2%, the value of K_s of soybean protein which hydrolysed by charcoal streptomyces proteinase at pH 8 and temperature (50°C) was 0.75% (Svendsen 1976). The value of K_S change slightly as pH and temperature varying, and the K_S in this study is 0.3664%, the result indicated that the 1398 neutral protease and fish protein has a better substrate affinity.

Determination of enzyme kinetic parameters The data in Figs. 1 and 2 were fitted according to the Eq. 6, then the parameter ("a" and "b") can be obtained and showed in following Table 1.

The result in Table 1 showed that parameter "b" change slightly and approximate to a constant, so the average of "b" was chose for its value (0.4511). Parameter "a" increased with the initial substrate concentration decreasing and the initial enzyme concentration

Table 1 The parameter ("a" and "b") in different conditions of enzymatic hydrolysis

$[S]_0$	$[E]_0$	$[E]_0/[S]_0$	a	b
3.6	0.20	0.0556	3.8663	0.4664
5.4	0.20	0.0372	2.2468	0.4537
7.2	0.20	0.0278	1.2623	0.4364
9.0	0.20	0.0222	1.2011	0.4344
4.5	0.16	0.0356	1.8782	0.4573
4.5	0.20	0.0444	2.7401	0.4669
4.5	0.24	0.0622	4.2304	0.4694
4.5	0.28	0.0533	3.2737	0.4483

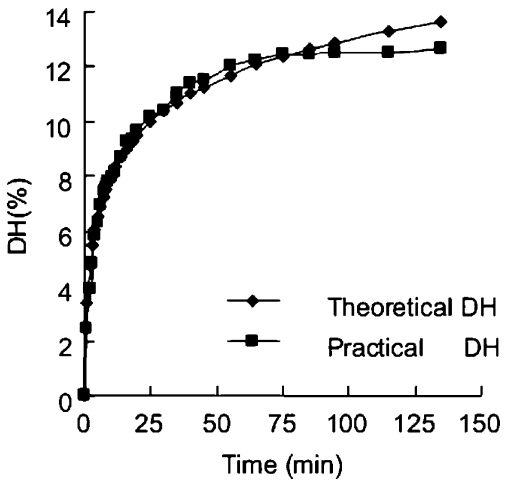

Fig. 5 The comparison of experimental DH and theoretical DH. The experiment was prepared in triplicates for each value (n=3)

increasing, thus parameter "a" and E_0/S_0 was linear relation and could be showed in Fig. 4 and in following equation:

$$a = 80.3360[E]_0/[S]_0 - 0.8098 \quad (10)$$

Therefore the model of enzyme kinetics equation was obtained:

$$DH = 2.2022 \ln\left[1 + \left(36.4806\frac{[E]_0}{[S]_0} - 0.3653\right)t\right] \quad (11)$$

The comparative result between theoretical hydrolysis degree and experimental hydrolysis degree showed in Fig. 5. This figure indicated that the experimental value is in line with the theoretical value better at a lower DH (<10%), when the degree of hydrolysis exceeded the value of 10%, there will be a deviation and the deviation increased with reaction time lengthening. The reason for this result may be the enzyme kinetics equation was derived under a lower degree of hydrolysis, so when the degree of hydrolysis is high comparatively, it can not correspond to the model of Eq. 11.

According to the Eq. 11, the critical enzyme concentration and the critical substrate concentration were obtained. The enzyme reaction could happen in this condition ($[E]_0 > \frac{0.3653[S]_0}{36.4806}$ and $[S]_0 < \frac{36.4806[E]_0}{0.3653}$).

Conclusions

In this study the 1398 neutrase was used for hydrolyzing fish protein, then analyzed the impact to the enzymolysis efficiency which were induced by the different initial substrate concentration and different initial enzyme concentration, and investigated the property of the enzymatic hydrolysis simultaneously. The result showed that the enzyme reaction velocity increase with the substrate concentration increasing when the substrate concentration was lower, but the comparative high substrate concentration will generate inhibition to enzyme. Michaelis constant was obtained and showed that the 1398 neutral protease and fish protein has a better substrate affinity. The enzymatic hydrolysis kinetics equation is derived by theory, by means of the verification to the enzymatic hydrolysis kinetics equation. it could see that the model is in line with the actual situation better at a lower hydrolysis degree. In the end, the critical enzyme concentration and the critical substrate concentration of enzyme reaction were obtained by deducing the enzyme kinetics equation.

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