Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Optimization of elastolysis conditions and elastolytic kinetic analysis with elastase from *Bacillus licheniformis* ZJUEL31410*

CHEN Qi-he†, HE Guo-qing†‡
(*Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China*)
†E-mail: qhchenzju@yahoo.com.cn; gqhe@zju.edu.cn
Received Aug. 3, 2005; revision accepted Nov. 13, 2005

Abstract: The solubilization of elastin by *Bacillus licheniformis* elastase cannot be analyzed by conventional kinetic methods because the biologically relevant substrate is insoluble and the concentration of enzyme-substrate complex has no physical meaning. In this paper we report the optimization of elastolysis conditions and analysis of elastolytic kinetics. Our results indicated that the hydrolyzing temperature and time are very important factors affecting elastolysis rate. The optimized conditions using central composite design were as follows: elastolysis temperature 50 °C, elastase concentration 1×10^4 U/ml, elastin 80 mg, elastolytic time 4 h. Investigation of the effects of substrate content, elastase concentration and pH was also revealed that low or high elastin content inhibits the elastolysis process. Increasing elastase improves elastin degradation, but high elastase may change the kinetics characterization. Alkaline environment can decrease elastin degradation rate and pH may affect elastolysis by changing elastase reaction pH. To further elucidate the elastolysis process, the logistic model was used to elastolysis kinetics study showing clearly that the logistic model can reasonably explain the elastolysis process, especially under lower elastase concentration. However, there is still need for more investigations with the aid of other methods, such as biochemical and molecular methods.

Key words: *Bacillus licheniformis*, Elastase, Elastolysis condition optimization, Elastolytic kinetics
doi:10.1631/jzus.2006.B0482 **Document code:** A **CLC number:** TS201.1

INTRODUCTION

Elastases are heterogeneous with differing substrate specificities and catalytic mechanisms. In fact, enzymes with elastolytic activity can be found in most of the major proteolytic families, including serine, thiol, aspartic, and metallo-enzymes. Despite the differences in catalytic mechanisms, all of these elastases share a common specificity for cleaving peptide bonds associated with hydrophobic or aromatic amino acids (Hall and Czerkawaki, 1961). Bacterial elastases belonging to the family of metalloproteinases have been known for many years. These proteinases require Zn atoms for activity and in contrast to the serine proteinases cleave peptide bonds on the amino-terminal side of the amino acid that determines specificity. Elastase from *Bacillus thermoproteolyticus* and *Streptomyces fradiae* are four and eight times more active than PPE (pig pancreatic elastase), respectively, making them some of the most potent elastolytic proteinases reported (Mecham *et al*., 1997).

Elastins are extracellular matrix proteins responsible for the resilience of tissues such as skin, arteries and lung and are insoluble, hydrophobic and extensively cross-linked protein forming fibers present in variable amounts depending on the tissue. Elastin displays extreme hydrophobicity and extensive cross-linking. Because of these properties, it is quite stable and persistent in tissues unless subjected to the catalytic activity of one or more of a group of proteinases, collectively termed elastases because of their ability to solubilize amorphous elastin (Mecham *et al*., 1997).

Solubilization of elastin is a complicated biologic process since the substrate is exceedingly insoluble and is also heterogeneous with respect to enzyme reaction

‡ Corresponding author
* Project (No. Y304203) supported by the Natural Science Foundation of Zhejiang Province, China

sites. Such a system is far more complex than the reaction of a proteinase with a soluble protein, and classical enzyme kinetics cannot be applied directly to give understanding of the elastolytic mechanisms. Elastin degradation with enzyme is very applicable and useful to food industry and daily use chemicals industry. Elucidation of elastolysis is beneficial for some diseases therapy related with elastin.

Optimization of batch bioreactors depends on the kinetics of the processes that can become extremely complicated for enzymatic hydrolysis of proteins. The large number of peptide bonds that are cleaved, both in parallel and in series, during the hydrolysis of most polymer molecules sets natural limitation to the possibility of estimating the basic kinetic parameters. This is a general problem in the establishing of kinetic models for the hydrolysis of macromolecular substrates. If the kinetic model is too simple, its inadequate representation of the true mechanism will limit its general applicability, while a complicated model cannot be analyzed statistically with sufficient precision by the often relatively crude kinetic experiments. According to Adler-Nissen, it is generally accepted that serine protease like alcalase, when acting on peptide bonds of protein, generally follow Michaelis-Menten kinetics (Adler-Nissen, 1986). However, other kinetics models such as logistic model were also used to describe proteolysis.

Considerable efforts were made to screen the elastase-producing strains, to study their pathogenic effect and their characterizations (Tsuzuki and Oka, 1965; Tsai *et al*., 1988; Sharon *et al*., 1997; Ozaki and Shiio, 1975). Reported studies on the reaction mode between elastin and elastase are rare (Hall and Czerkawaki, 1961). The reaction was found to be a complex process of at least three steps (Heather *et al*., 1999). The mechanism of the reaction is still under investigation at present. There are many studies focused on the identification and purification of elastase-producing strains, but few on elastolysis. In a previous work, a new elastase was isolated from *Bacillus licheniformis* ZJUEL31410 to study its cultivation conditions and fermentation kinetics (Chen *et al*., 2002; 2004); this enzyme was a protease with very high elastolytic activity, so it is interesting to further study its structural and functional relationships, including the difference in substrate specificity. In the present work, elastolysis conditions were optimized and the elastolysis kinetics model was preliminarily constructed.

MATERIALS AND METHODS

Materials

Elastase was made from *Bacillus licheniformis* ZJUEL31410 (stored in my lab) culture liquid with graded salts precipitation (45%~70%). The elastase activity after precipitation was 20000 U/ml (Chen *et al*., 2003). Crude elastin was produced by the method of Partridge and Davis (1948). Standard elastin was bought from Sigma Company (USA). Coomaas brilliant blue and casein were bought from Shanghai Biochemical Co. Ltd. (China). Congo-red elastin from bovine neck ligament was purchased from Sigma Co. Ltd. (USA). Other chemical agents were analytical grade (Hangzhou, China).

Microorganism and media

Bacillus licheniformis ZJUEL31410 was isolated and mutated by Zhejiang University, China. This strain was maintained on LB (g/L) slants (peptone 6, yeast extract 2, beef extract 4, NaCl 5, agar 2). The medium for the inoculum contained the above components in addition to the agar.

Assay methods

1. Enzyme assay

Elastolytic activity was assayed by the colorimetric method of Sachar (1955). Enzyme preparation was incubated with 20 mg of congo-red elastin in 2 ml of 0.2 mol/L boric acid buffer (pH 7.4) with shaking for 20 min at 37 °C. The reaction was stopped by adding 2 ml of 0.7 mol/L sodium phosphate buffer (pH 6.0), and immediately filtered. Absorbency of the filtrate was read at 495 nm against the control (no enzyme). One unit of elastase activity was defined as the amount of enzyme required to solubilize 20 mg elastin-congo red under the tested conditions.

2. Enzyme preparation

Crude elastase and purified elastase were prepared by such method. Briefly, *Bacillus licheniformis* ZJUEL31410 was aerobically cultured in a medium containing glucose and casein and corn steep flour, at 37 °C for 30 h. Ammonium sulfate precipitation (40%~70%) of the culture fluid was performed to

obtain the partially purified enzyme, and this fraction was then further purified using Sephadex G-100 (Pharmacia, Co. USA) column chromatography (Chen *et al*., 2003).

3. Protein content

The protein hydrolysate content was determined by Bradford (1976) method, using bovine serum albumin (BSA) as the standard. The content of amino of elastin was measured by the method of Lahl (1994).

4. Experimental methods

In the optimization process, the hydrolyzing factors were optimized with statistical design. Then under this optimized condition, the degradation process was investigated after studying the effect of pH, temperature and enzyme concentration on the elastolysis process. In the experiment, the hydrolyzing rate was calculated by the following equation:

$$DH=S_N/T_N\times 100\% = \frac{(OD_{595}+0.0012)\times 0.5140\times 100\%}{0.0987\times W_{\text{substrate}}},$$

where DH is degree of hydrolysis; S_N is the soluble nitrogen; T_N is the total nitrogen; OD_{595} is the absorbency density at wavelength 595 nm; $W_{\text{substrate}}$ is elastin weight added.

Experimental designs

In this work the experimental sets were designed with SAS software and the results were analyzed by SAS software also. Response surface methodology (RSM) is an excellent experimental strategy for seeking optimal conditions for multi-variables system, and have been successfully employed for optimizing the medium composition and operating conditions in many bioprocess (Ma and Ooraikul, 1986). We used the central composite design based on previous experiment to optimize the following factors. The experiment factor level design is shown in the Table 1 in the form of both coded values and natural values. The correspondence between these values can be calculated by: $X_i=(x_i-x_{i0})/\delta_i$, where X_i is the coded value, x_i is the corresponding natural value, x_{i0} is the natural value at the center of the domain, and δ_i is the increment of x_i corresponding to 1 unit of X_i.

A full second-order polynomial model obtained by a multiple regression technique using the SAS package (SAS Institute, Cary, NC, USA) was adopted to describe the response surface. For three factors, the model obtained is expressed as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{12}X_1X_2+b_{13}X_1X_3 +b_{14}X_1X_4+b_{23}X_2X_3+b_{34}X_3X_4+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{44}X_4^2,$$

where Y is the measured response (elastase activity in this study), b_0 is the intercept term, b_1, b_2, b_3 and b_4 are linear coefficients, b_{12}, b_{13}, b_{14}, b_{23} and b_{34} are the interactive coefficient, b_{11}, b_{22}, b_{33} and b_{44} are quadratic coefficients, and X_1, X_2, X_3 and X_4 are coded independent variables.

RESULTS AND DISCUSSION

Optimization of elastolysis conditions with *Bacillus licheniformis* elastase

The degradation conditions are important for proteolysis process. In this part, the effects of elastolytic temperature, elastase activity, elastin content and elastolysis time on elastolysis rate were investigated and optimized by using response surface methodology. The experimental design and its results are shown in Table 1.

Based on the analysis results shown in Table 2 and Table 3, it was apparent that the second model is very significant for this response (α=0.00001). And the model is also very stable and appropriate (α=0.0180). The regression results (Table 2 and Table 3) indicated that the most important factors for elastolysis were temperature (X_1), substrate (X_3) and elastolytic time (X_4); elastase concentration (X_2) is not the most insignificant for elastolysis. This second model was determined mainly by the quadratic term of four factors. Based on the above results, the enzyme concentration has little impact on elastin degradation; hydrolyzing time, temperature and elastin content were the most effective factors for elastolysis process.

Fig.1 indicated the effect of four factors on elastolysis rate. The results of response surface analysis showed that at the point of maximal elastolysis rate, the elastolysis conditions were as follows: elastolysis temperature 50 °C, elastase activity 10000 U/ml, elastin content 80 mg, elastolytic time 4 h. A verifying experiment conducted at the combination of optimal elastolytic conditions showed that the selected model is very suitable for explaining the actual elastolysis process.

Table 1 Central composite design and the responding results for elastolysis conditions

Runs	Temperature X_1	Elastase con. X_2	Substrate con. X_3	Elastolytic time X_4	OD_{595}	DH (%) Y (OD_{595})
1	1	1	1	1	0.182	32.83
2	1	1	1	−1	0.188	33.90
3	1	1	−1	1	0.077	30.83
4	1	1	−1	−1	0.090	35.95
5	1	−1	1	1	0.169	30.50
6	1	−1	1	−1	0.121	21.90
7	1	−1	−1	1	0.073	29.25
8	1	−1	−1	−1	0.089	35.60
9	−1	1	1	1	0.159	28.71
10	−1	1	1	−1	0.109	19.75
11	−1	1	−1	1	0.094	37.53
12	−1	1	−1	−1	0.067	26.89
13	−1	−1	1	1	0.162	29.24
14	−1	−1	1	−1	0.054	9.90
15	−1	−1	−1	1	0.100	39.90
16	−1	−1	−1	−1	0.093	37.14
17	−2	0	0	0	0.162	40.21
18	2	0	0	0	0.221	54.75
19	0	−2	0	0	0.206	51.05
20	0	2	0	0	0.180	44.65
21	0	0	−2	0	0.064	64.26
22	0	0	2	0	0.206	29.17
23	0	0	0	−2	0.051	12.86
24	0	0	0	2	0.061	15.33
25	0	0	0	0	0.199	49.33
26	0	0	0	0	0.230	56.97
27	0	0	0	0	0.204	50.56
28	0	0	0	0	0.211	52.28
29	0	0	0	0	0.208	51.54
30	0	0	0	0	0.236	58.44
31	0	0	0	0	0.194	51.00

$X_1=(x_1-50)/10$; $X_2=(x_2-20000)/10000$; $X_3=(x_3-80)/30$; $X_4=(x_4-3)/1$

Table 2 Results of ANOVA for parameters of four factors against elastolysis rate (OD_{595})

Source	Sum of square	Degree of freedom	Mean square	F-value	Significant level
X_1	0.0072	1	0.0072	12.27766	0.00294
X_2	0.0003	1	0.0003	0.47661	0.49986
X_3	0.0553	1	0.0553	94.17236	0.00000
X_4	0.0050	1	0.0050	8.58966	0.00979
X_1^2	0.0068	1	0.0068	11.64804	0.00356
X_2^2	0.0063	1	0.0063	10.79094	0.00466
X_3^2	0.0398	1	0.0398	67.78160	0.00000
X_4^2	0.1317	1	0.1317	224.17510	0.00000
X_1X_2	0.0006	1	0.0006	1.07530	0.31517
X_1X_3	0.0060	1	0.0060	10.28240	0.00550
X_1X_4	0.0048	1	0.0048	8.15471	0.01145
X_2X_3	0.0038	1	0.0038	6.43422	0.02199
X_2X_4	0.0012	1	0.0012	2.01596	0.17484
X_3X_4	0.0057	1	0.0057	9.67768	0.00672

Table 3 ANOVA results of second-order model from central composite design (OD_{595})

Sources	Sum of square	*DF*	*MS*	*F*-value	Significant level
Regression	0.1051	14	0.0075	12.80000	0.00001
Residual	0.0094	16	0.0006		
Lack of fit	0.0079	10	0.0008	3.24900	0.01750
Error	0.0015	6	0.0002		
Total	0.1145	30			

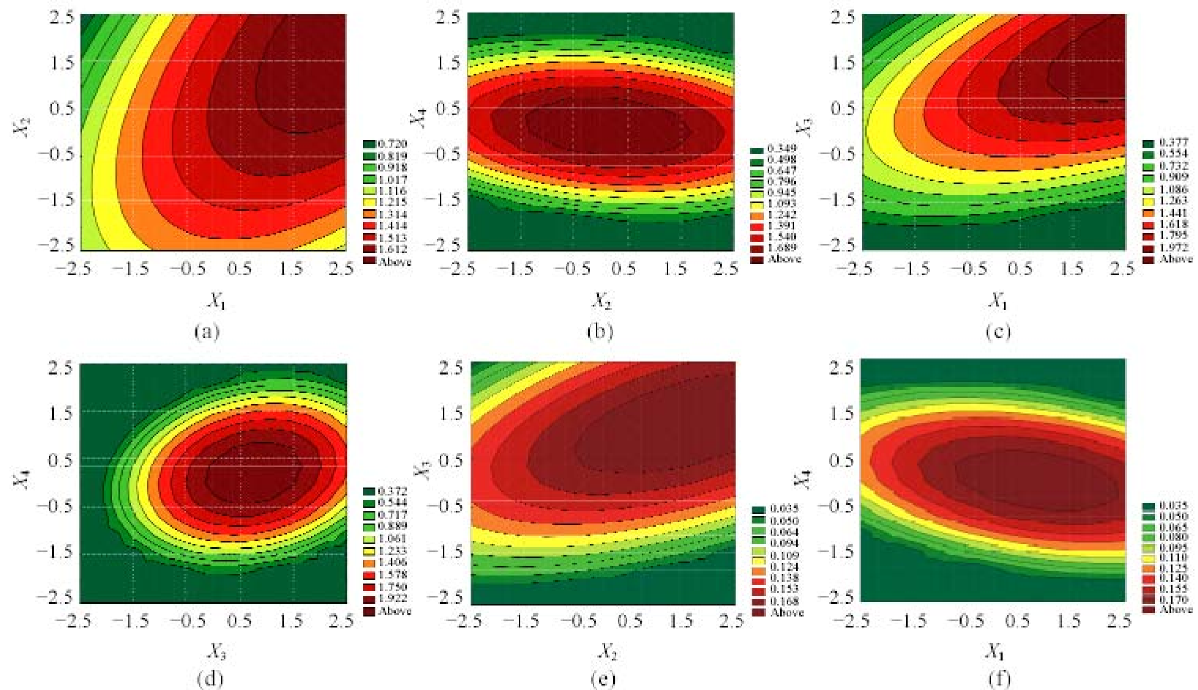


Fig.1 The contour plot of elastolysis temperature (X_1), elastase activity (X_2), substrate concentration (X_3) and elastolytic time (X_4) affect elastolysis rate for *Bacillus licheniformis* ZJUEL31410 elastase. (a) Effect of X_1 and X_2 against elastolysis rate; (b) Effect of X_2 and X_4 against elastolysis rate; (c) Effect of X_1 and X_3 against elastolysis rate; (d) Effect of X_3 and X_4 against elastolysis rate; (e) Effect of X_2 and X_3 against elastolysis rate; (f) Effect of X_1 and X_4 against elastolysis rate

Effect of different *Bacillus licheniformis* elastase activity on elastolysis process

The above results showing that elastase concentration had little effect on elastolysis mean that elastase did not play certain role in elastin degradation. Because of the dissolubility of elastin in elastolysis process, the elastolysis of elastin is a very complex system, especially for elastase active site and elastin reaction site. This reaction system is more complex than that of soluble substrates and enzymes. Consequently, it is not easy to explain the reaction mechanism by using classical kinetics.

For these reasons, this part mainly discusses the effect of enzyme concentration on elastolysis process. Fig.2 shows the experimental results. It was found that the elastase concentration had significant impact on elastolysis rate. However, elastase activity had no effect on elastolysis mode. With increasing reaction time, the elastolysis rates were not the same among different enzymes concentrations. However, elastase concentration had no significant impact on elastin degradation mechanism. According to the result of Fig.2, it was difficult to describe and explain elastolysis process with using the classical kinetics model such as Monod equation. Under the low elastase concentrations, the elastolysis kinetics demonstrated the first-order type, or else the opposite. Under high elastase concentrations, the change point all appeared at the reaction time 180 min of the elastolysis curves. During the reaction time from 180 to 240 min, the reaction rate decreased slowly, and after that the degradation rate increased rapidly.

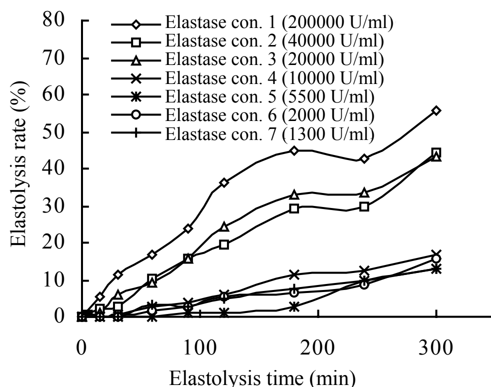


Fig.2 Elastolysis course comparison under different *Bacillus* elastase

Impact of different substrate content on elastolysis process

The effect of different elastin content on elastolysis process was investigated in this part. The experimental result of Fig.3 shows that adding elastin significantly affected elastolysis process. High or low elastin amount greatly inhibits elastin degradation. It was supposed that lower substrate amounts likely are not enough for enzyme absorption and degradation; and that too high substrate amount may possibly have inhibitory effect on elastolysis. This phenomenon had been experimented on and verified in the research on pig pancreatic elastase. The phenomenon's causative reason was that PE (pancreatic elastase) has two substrate-absorbing active sites. With the first site having higher substrate-absorbing capacity that requires lower substrate content than the second site, so its reaction kinetics conforms to the general kinetics system; with the second site having lower substrate-absorbing capacity that requires more substrates in the reaction process, which certainly inhibit the first site's decrease in the elastolysis rate (Heather *et al.*, 1999).

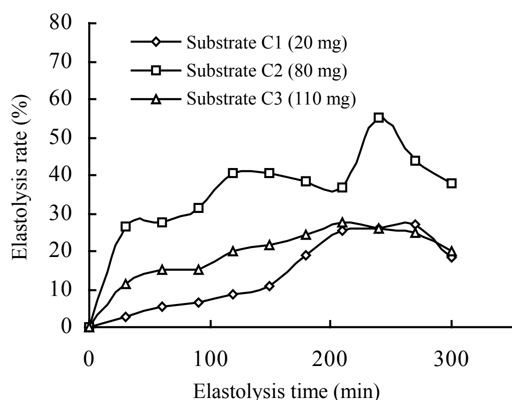


Fig.3 Elastolysis course comparison under different elastin content

The maximal elastolysis rate was observed at the reaction time 250 min for three treatments. But before 60 min of reaction time, the reaction kinetics of these three substrates showed first-order mode; after that the reaction kinetics did not conform to the classical kinetics model. With increasing substrate concentration, the reaction process is not well explained by the classical kinetics model, such as Michaelis-Menten kinetics.

Effect of different pH on elastolysis process

At the optimal point of pH, all reactions showed the maximal reaction rate. When this point is higher or lower than the optimal point, the maximal reaction rate both will decrease. pH is an important factor that be helpful in providing much more proofs for explaining its reaction mechanism. Considering this point, the effect of pH on elastolysis rate was researched. Experimental results are shown in Fig.4.

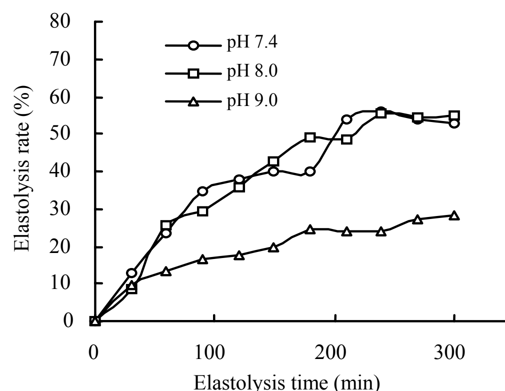


Fig.4 Effect of pH on elastolysis rate for *Bacillus* elastase

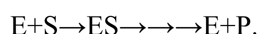
From Fig.4, it is apparent that pH has significant impact on elastolysis process. The elastolysis rate decreases with increasing pH; and at pH 9.0 was apparently lower than that at pH 7.4 and 8.0. The maximal reaction rate appeared at 240 min under pH 7.4 and pH 8.0. The elastolysis curves were the same before the 240 min reaction time under pH 7.4 and pH 8.0; after that odd change occurred. High pH may likely cause the enzyme to denature or partially stop reacting veri-

fied in some researches (Du *et al*., 1991). For this elastase studied, the optimal reaction pH was found to be 7.4 in my previous study (Chen *et al*., 2003), which accorded with the elastolysis change in this study.

Study on the kinetics model of elastolysis

Elastin is a very dissolving protein that is not easily degraded by proteases and other methods. So the attack process of elastase on elastin and elastolysis could not be described and explained by the classical hydrolyzing kinetic law. So the degradation mechanism was not easy to be understood. With using the new technology and experimental methodology, the elastolysis mechanisms will be gradually understood, so elastolytic kinetics can possibly be investigated.

A simplified model of elastin solubilization can be considered to proceed according to the equation:



$$E+S \rightarrow ES \rightarrow \rightarrow \rightarrow E+P.$$

Lonky and Wohl (1983) postulated the existence of two types of binding sites for HLE (human leukocyte elastase) on elastin. The mechanism of binding to the first class of site was thought to be electrostatic, which was permanent but did not result in catalysis. The second class was thought to be located in the alanine-rich hydrophobic cross-linked areas of elastin and to lead to catalysis (Heather *et al*., 1999). Based on the studies reported, the logistic kinetics model was generally adopted to describe the proteolysis process, especially for macromolecular substrates (Mecham *et al*., 1997). Fig.5 and Table 4 show the experimental results of the kinetics model analysis, respectively. Results clearly showed that the kinetics model selected was suitable for explaining the elastolysis process as the enzyme concentration decreased. The statistical data in Table 4 apparently showed that the logistic model was very significant and very appropriated (P=0.001, R^2>0.93). The logistic model is not good for explaining the actual elastolysis process as increasing the elastase concentration, which still is under investigation. The experimental results were also rechecked in Fig.5. From the simulation figures of real value and predicted value, it was found that the logistic model could better describe the elastolysis process when enzyme concentration is low. Elastolysis is a very complex process that is not yet fully understood. Consequently, research on the kinetics model was obviously not easy. However, adopting the simple kinetics model is likely appropriate for explaining its hydrolyzing process and its elastolysis mechanism to some degrees.

CONCLUSION

Elastin is the extracellular matrix protein that imparts elastic recoil to tissues. Its cross-linked nature and extreme hydrophobicity make it one of the most stable proteins in the body. This paper first discussed the optimization of elastolysis conditions and the elastolysis kinetics using *Bacillus licheniformis* elastase. Experimental results indicated that the optimized elastolysis conditions were as follows: temperature 50 °C, elastase activity 1×10^4 U/ml, elastin 80 mg, elastolysis time 4 h. Hydrolyzing temperature and substrate amounts were found to be the most important factors affecting elastolysis. Different elastase concentrations had significant impact on the elastolysis process. At higher elastase concentration, the elastolysis process cannot suitably be explained by logistics model. The high and low substrate addition both inhibits the elastolysis process. However, under three substrates the maximal elastolysis rate occurred at 250 min. It implied that elastolysis might have a special mechanism that determines its elastolysis process. Based on the above studies, it is supposed that the binding time of elastase and elastin

Table 4 Analysis results for logistic model of elastolysis under different *Bacillus* elastase activity

Elastase con.	Logistic equation	Significant level (α)	R^2
1	X_2=66.9326/(1+exp(2.0054−0.050122X_1))	0.00018	0.9428
2	X_2=49.6763/(1+exp(2.3651−0.026594X_1))	0.00004	0.9653
3	X_2=41.8260/(1+exp(2.4346−0.017815X_1))	0.00018	0.9435
4	X_2=38.7245/(1+exp(2.7929−0.026562X_1))	0.00002	0.9719
5	X_2=16.7551/(1+exp(3.0898−0.020048X_1))	0.00001	0.9778
6	X_2=14.3158/(1+exp(6.8225−0.031428X_1))	0.00000	0.9942

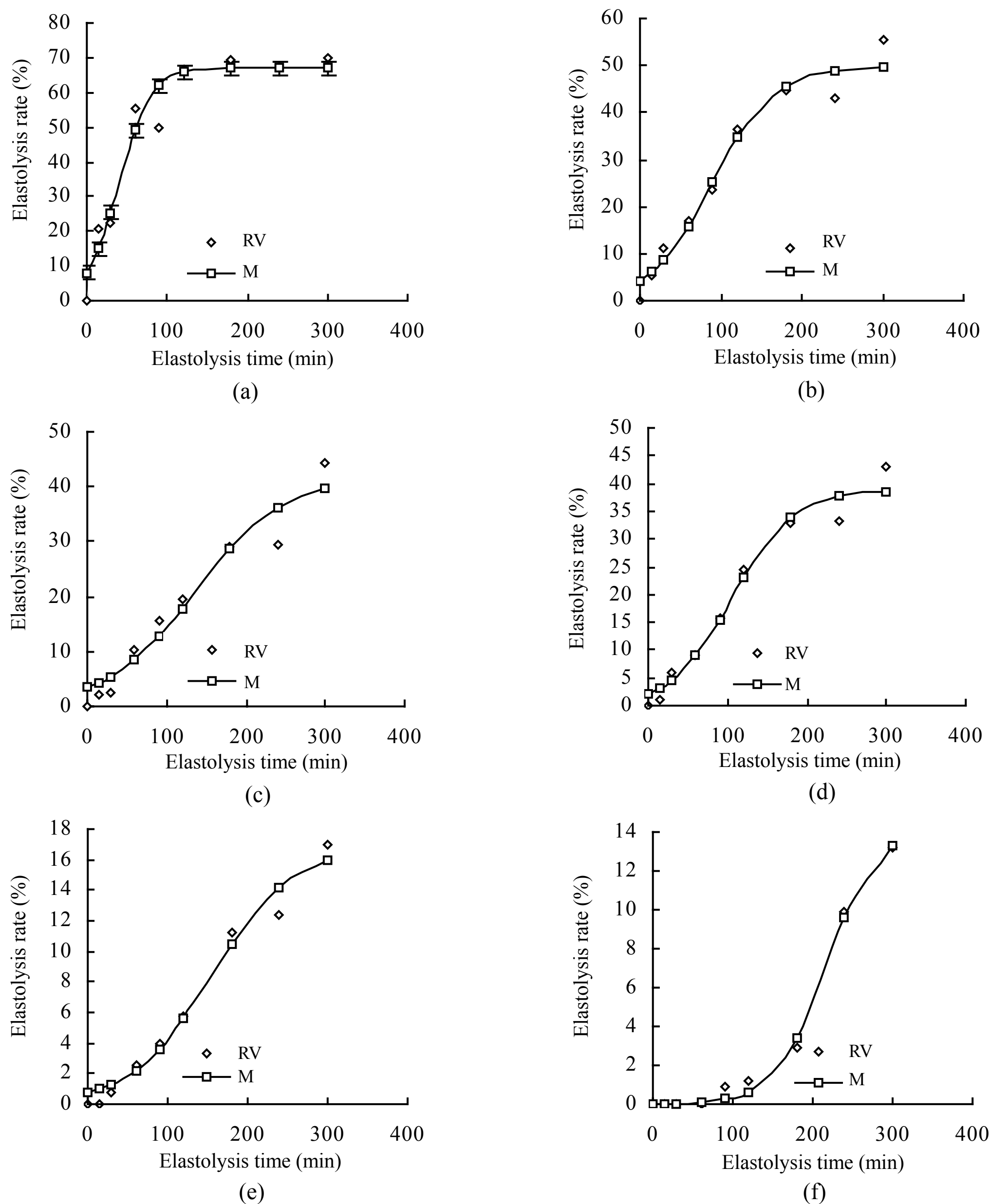


Fig.5 Comparison curves of the modeling value (M) and the experimental value (RV) for elastolysis under different elastase concentrations (logistic model). (a) 200000 U/ml; (b) 40000 U/ml; (c) 20000 U/ml; (d) 10000 U/ml; (e) 5500 U/ml; (f) 2000 U/ml

is not affected by the mechanism, but the binding rates of elastin and elastase were changed in this case. Baici (1992)'s studies showed that following initial complex formation there is a local structural change in elastin that alters the enzyme-substrate interaction and allows catalysis to proceed. It is supposed that elastin structural change is very important for elastolysis to proceed. In this work the elastolytic kinetics model was also investigated. Heather *et al.*(1999) attempted to apply elements of classical Michaelis-Menten kinetics to the study of elastolysis. Some quantitatively useful measures were obtained, but their results had no clear-cut physical meanings because empirical fits of their model to experimental results were acceptable only for selected reaction conditions (Heather *et al.*, 1999). Consequently, the

logistic model was used to describe and explain the elastolysis process. The experimental results showed that this model explained well the real process of elastin degradation, especially under low enzyme concentration. However, some inroads have to be made into the theoretical understanding of enzyme kinetics in heterogeneous reaction systems when one or more reactants are spatially constrained. So much more studies have to be conducted in this field.

References

Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Protein. Elsevier Applied Science Publishers, London.

Baici, A., 1992. Current Topics in Rehabilitation. *In*: Grassi, C., Travis, J., Casali, L., Luisetti, M. (Eds.), Biochemistry of Pulmonary Emphysema. Spring-Verlag, Berlin, p.81-91.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**(1-2):248. [doi:10.1016/0003-2697(76)90527-3]

Chen, Q.H., He, G.Q., Molhtar, A.M.A., 2002. Optimization of medium composition for the production of elastase by *Bacillus* sp. EL31410 with response surface methodology. *Enzyme Microb. Technol.*, **30**(5):667-672. [doi:10.1016/S0141-0229(02)00028-5]

Chen, Q.H., He, G.Q., Wu, Y.L., 2003. Screening of elastase-producing strains and primary studies on elastase fermentation conditions. *Journal of Zhejiang University (Agriculture and Life Science)*, **59**(1):59-62 (in Chinese).

Chen, Q.H., He, G.Q., Schwarz, P., 2004. A kinetic model for elastase production by *Bacillus* sp. EL31410. *Journal of Agricultural and Food Chemistry*, **52**(11):3356-3359. [doi:10.1021/jf0303161]

Du, J.Z., Shu, B.G., Wei, X.C., 1991. Enzyme Structure and Reaction Mechanism. Peking University Press, Beijing, p.104-180 (in Chinese).

Hall, D.A., Czerkawaki, J.W., 1961. The reaction between elastase and elastic tissue. *Biochem. J.*, **80**:128-136.

Heather, M.M., Howard, G.W., Caroline, A.O., Robert, A.S., Edward, J.C., 1999. Interaction between leukocyte elastase and elastin: quantitative and catalytic analyses. *Biochimica et Biophysica Acta*, **1430**(2):179-190.

Lahl, R., 1994. Enzymatic production of protein hydrolysates for food uses. *Food Technol.*, **138**:68-71.

Lonky, S.A., Wohl, H., 1983. Regulation of elastolysis of insoluble elastin by human leukocyte elastase: stimulation by lysine-rich ligands, anionic detergents, and ionic strength. *Biochemistry*, **22**(15):3714-3720. [doi:10.1021/bi00284a027]

Ma, A.Y.M., Ooraikul, B., 1986. Optimization of enzymatic hydrolysis of canola meal with response surface methodology. *J. Food Proc. Preserv.*, **10**:99-113.

Mecham, R.P., Broekelmann, T.J., Fliszar, C.J., Shapiro, S.D., Welgus, H.G., Senior, R.M., 1997. Elastin degradation by matrix metalloproteinases. *J. Biol. Chem.*, **272**(29):18071-18076. [doi:10.1074/jbc.272.29.18071]

Ozaki, H., Shiio, I., 1975. Purification and properties of elastolytic enzyme from *Flavobacterium immotum*. *J. Biochem.*, **77**:171-180.

Partridge, S.M., Davis, H.F., 1948. The chemistry of connective tissues. *Biochemical J.*, **43**:387.

Sachar, L.A., 1955. Photometry method for estimation of elastase activity. *Proc. Soc. Expeti. Biol. Med.*, **90**:5323-5325.

Sharon, S.R., Jennifer, L.R., Bryan, N.B., 1997. Identification of an elastolytic protease in stationary phase culture filtrates of *M. tuberculosis*. *FEMS Microbiology*, **151**(1):59-64.

Tsai, Y.C.H., Jung, R.Y., Lin, S.F., 1988. Production and further characterization of an alkaline elastase production by alkalophilic *Bacillus* strain YaB. *Appl. Environm. Microbial.*, **54**(1):3156-3161.

Tsuzuki, H., Oka, T., 1965. *Pseudomonas aeruginosa* elastase: isolation, crystallization and preliminary characterization. *J. Biol. Chem.*, **8**:3295-3303.

Editors-in-Chief: Pan Yun-he & Peter H. Byers
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online), monthly

Journal of Zhejiang University
SCIENCE B

www.zju.edu.cn/jzus; www.springerlink.com
jzus@zju.edu.cn

***JZUS-B* focuses on "Biomedicine, Biochemistry & Biotechnology"**

JZUS-B online in PMC: http://www.pubmedcentral.nih.gov/tocrender.fcgi?journal=371&action=archive