Bot. Bull. Academia Sinica 28: 131-138 (1987)

KINETICS OF PAPAIN IMMOBILIZED ON CHITOSAN BY MULTIPLE POINT ATTACHMENT[1,2]

JEI-FU SHAW, REY-CHANG CHANG and YNG-JIIN WANG[3]

Institute of Botany, Academia Sincia
Nankang, Taipei, Taiwan 11529, Republic of China

(Received October 17, 1986; Accepted December 16, 1986)

Abstract

Papain was coupled to chitosan by multiple point attachment. The enzyme coupled was 83.8 mg per gram of chitosan and the relative specific activities of the immobilized papain to soluble enzyme with casein and BAEE as substrates were 63% and 65%, respectively. The optimal pH of immobilized papain (pH 8.0) was one unit higher than that of soluble papain and the activity was less sensitive to pH change. The immobilized papain also had higher pH stability than soluble form. The immobilized papain had higher apparent Km values with either casein or BAEE as substrate and the deviations appeared to be more pronounced with casein as substrate. The optimal temperature was greatly increased from 52°C to 68°C as a result of immobilization and the activity of the immobilized papain was less sensitive to temperature change. The multiple attachment enzyme (CN-EDC-GA) was 3.8 times more stable than the traditional single attachment enzyme (CN-GA) in the batch type operation with casein as substrate at 45°C. From the Arrhenius plot, it was found that the isokinetic temperature was -6°C and the multiple attachment offered high temperature stability. The energy of activation (Ea), activation entropy (ΔS^*) and activation enthalpy (ΔH^*) for operational denaturation of CN-EDC-GA system were 17%, 24% and 17% lower than those of CN-GA system at 45°C.

Key words: Chitosan; papain; multiple attachment; immobilization; kinetics.

Introduction

Chitin is an economical support for enzyme immobilization (Riccardo and Muzzarelli, 1980). It can be produced from the agarowaste crab shell and the estimated production of crab shell in Taiwan was 500 tons per year, which cor-

[1] Paper No. 323 of the Scientific Journal Series, Institute of Botany, Academia Sinica, Taipei, Taiwan 11529, Republic of China.

[2] This work was supported by a research grant from National Science Council, Republic of China to J.F. Shaw.

[3] Department of Biomedical Engineering, Yang-Ming Medical College, Taipei, Taiwan, Republic of China.

responded to 10-100 tons of chitin (Chang, 1982). Chitosan can be produced from chitin by alkaline deacetylation (Knorr, 1984). Most of the previous utilization of chitin as a support for enzyme immobilization was simply coupling the enzyme to chitosan by glutaraldehyde cross linking (Riccardo and Muzzarelli, 1980). Recently we found that multiple attachment of lipase on chitosan offered great stability against denaturation (Chang and Shaw, 1987). Papain is widely used for chill-proofing of beer (Bass and Cayle, 1975) and synthesis of peptides (Fruton, 1982). It has been immobilized on chitosan by simple glutaraldehyde cross linking (Finley *et al.* 1977), however, no detailed kinetic properties were reported. In the present work, we further improved the papain stability by multiple point attachment on chitosan and studied the kinetics in some detail.

Materials and Methods

Materials

Chitosan, papain (type 2, 1.6-2.8 units per mg solid), casein and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma Co. Benzoyl L-arginine ethyl ester (BAEE) and glutaraldehyde were obtained from E. Merk Co. All other chemicals were of reagent grade.

Immobilization of Papain

The chitosan was dissolved in 0.1 N HCl solution and adjusted to pH 6.0 by NaOH. This soluble chitosan (100 ml, 0.3%) was coupled to papain (0.15 g) by EDC (0.2%) at 4°C. This complex was cross linked by glutaraldehyde (0.5%) to form insoluble papain which was linked to chitosan by multiple point attachment.

Enzyme Assay for Immobilized Papain

The papain was assayed with either casein or BAEE as substrate. Immobilized protease was put in a covered sintered glass (1G4). After the addition of 4 ml of prewarmed (20-60°C) casein solution (0.2% in 0.1M buffer solutions as required in given experiment). Sodium acetate buffer was the buffer used for pH 5.0-5.5, sodium phosphate for pH 5.8-8.0 and sodium borate for pH 8.1-10.0. The reaction mixture was incubated in a shaking water bath at the temperature as indicated in the legend. Then the enzyme was removed by filtration and 1 ml of filtrate was used to determine the absorbance at 275 nm by TCA method (Murachi, 1970). One protein hydrolysis unit is defined as the increase of absorbance of one mole of tyrosine at 275 nm per min.

In the case of using BAEE as the substrate, the reaction mixture containing immobilized protease, 5 ml BAEE (0.05M) and 1 ml KCl (0.1M) was incubated in a water bath at 30°C. Sodium hydroxide (0.05 M) was added to maintain constant

pH at 7.0 reaction proceeded for 2 minutes. From the amount of NaOH consumed, the protease activity can be calculated.

Determination of apparent Km and apparent Vm

These kinetic values were determined by Lineweaver-Burk plots. The initial velocities of the immobilized enzymes were determined by enzyme assay as described above with casein as substrate (0.025%-0.5% casein in 0.2M phosphate buffer, pH 7.0). The amount of enzyme was 6 mg.

Results and Discussion

The enzyme coupled was 83.8 mg enzyme per gram of chitosan. The relative specific activities of the immobilized papain to soluble enzyme with casein and BAEE as substrate were 63% and 65%, respectively. The optimal pH of immobilized papain was 8.0, which was one pH unit higher than that of soluble papain, when the enzyme was assayed at 37°C with casein as substrate (Fig. 1). The increase of optimal pH was possibly due to the steric hindrance of the support which obstructed the diffusion of protons to the solution (Ax'en *et al.*, 1967; Srere and Mosbach, 1974). The activity of immobilized papain was also less sensitive to pH change. As shown in Fig. 2, the immobilized papain had higher pH stability than soluble papain when enzymes were incubated in various pH buffers at 37°C for half an hour and then assayed for residual activity.

Figure 3 showed that the apparent Km and Vm of immobilized papain can be obtained from double reciprocal plot. These values were listed in Table 1. The

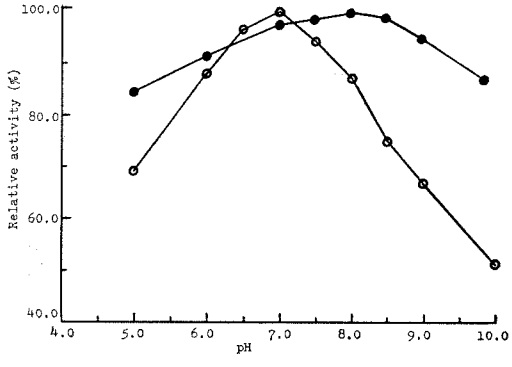

Fig. 1. pH behavior for various papain. The enzymes were assayed at 37°C with casein as substrate.
○-○: soluble; ●-●: CN-EDC-GA

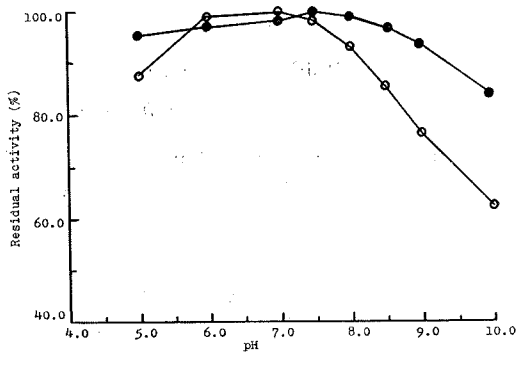

Fig. 2. pH stability of soluble and immobilized papain at 37°C. They were incubated in various pH buffers for 30 min and then assayed for the residual activity with casein as substrate at pH 8.0.
○-○: soluble; ●-●: CN-EDC-GA

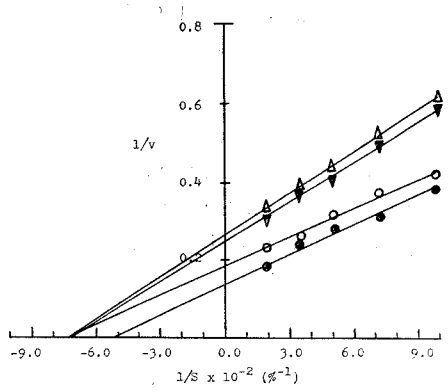

Fig. 3. Lineweaver-Burk plots for the hydrolysis of casein by CN-EDC-GA papain at various temperatures. The substrate concentration (S) was expressed as % casein solution and the initial velocity was expressed as μ mole/min/6 mg papain.
△-△: 37°C; ▼-▼: 42°C;
○-○: 47°C; ●-●: 52°C

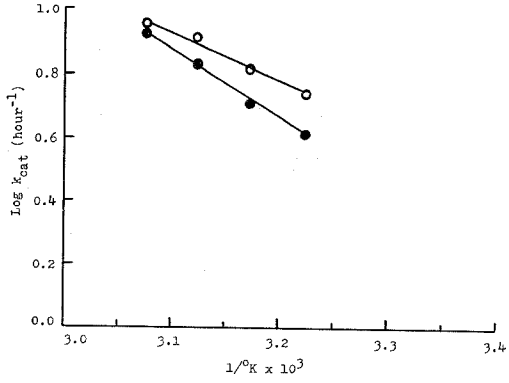

Fig. 4. Arrhenius plots of log k_{cat} vs. reciprocal of temperature in °K for various papain in caseinolysis reaction at pH 8.0.
○-○: soluble; ●-●: CN-EDC-GA

immobilized enzyme had higher apparent Km values at all the temperature tested with either casein or BAEE as substrate. The deviations appeared to be more pronounced with casein as substrate; for example, the Km increased 17% and 45% with BAEE and casein as substrates respectively at 37°C. These effects can be interpreted by the steric hindrance of carrier which caused diffusional limitation of substrate around the microenvironment of immobilized enzymes. Since immobilized enzyme particles are enveloped by a quiscent layer of solvent (Nernst

Table 1. *Kinetic parameters of soluble and immobilized papain at various temperature*

The enzymes were assayed at pH 8.0 with casein and BAEE as substrates.

System		Temperature °C								Ea (Kcal/mole)
		37		42		47		52		
		Vm[a]	Km[b]	Vm	Km	Vm	Km	Vm	Km	
Soluble	Casein	5.53	0.095	6.57	0.100	8.13	0.116	8.90	0.120	6.6
	BAEE	2.4	3.5	2.8	3.8	3.2	3.9	3.5	4.0	5.4
CN-EDC-GA	Casein	3.85	0.138	4.23	0.140	5.63	0.148	7.11	0.200	8.5
	BAEE	1.5	4.1	1.7	4.0	2.1	4.8	2.3	4.9	5.5

[a] Vm = μ mole/min/6 mg papain.
[b] Km is expressed as % casein solution or mM concentration of BAEE.

layer) which leads to substrate concentration gradient, higher substrate concentrations are required to saturate the immobilized enzymes compared with soluble enzymes form (Kilara and Shahani, 1979). The Km values of immobilized enzymes are generally higher than those of soluble form. The small substrate BAEE presumably had less diffusional limitation and thus had smaller deviation of Km than larger substrate casein. Both Vm and Km values in soluble and immobilized papain increased as the temperature increased from 37°C to 52°C (Table 1). The increase of Km was due to the weakening of ionic interaction between protease and its substrate (Goldstein *et al*, 1964) since $Vm = k_{cat} \times e$, where e represents enzyme concentration, the energy of activation Ea can be obtained from Arrhenius plots (Fig. 4). As shown in Table 1, the energies of activation for immobilized papain were higher than those of soluble papain. Again reactions with BAEE as substrate had less deviation than those with casein as substrate.

As shown in Fig. 5, the optimal temperature was greatly increased from 52°C to 68°C as a result of this immobilization and the activity of the immobilized enzyme was less sensitive to temperature change. To demonstrate the effect of multiple point attachment, the operational stabilities of papain immobilized on chitosan by direct glutaraldehyde attachment (CN-GA) and by multiple attachment (CN-EDC-GA) were compared. The denaturation of both immobilized papains in batch type operation followed first order kinetics (Fig. 6). This suggested that autodigestion of papain colud be eliminated by immobilization. From the equation $\ln A_t/A_0 = -k \times t$, where A_0 and A_t represent the initial enzyme activity and residual enzyme activity at time t respectively, the apparent denaturation rate constant k could be obtained from the slope (Table 2). From $\ln k = A - Ea/R$

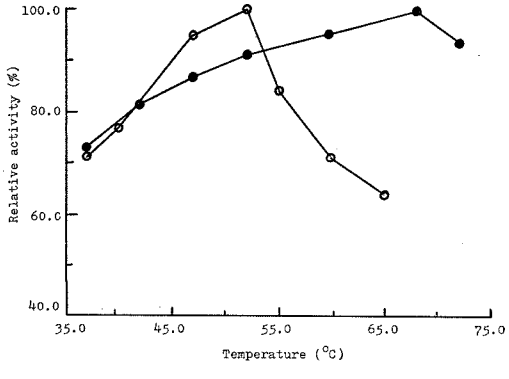

Fig. 5. Temperature behavior for various papain. The enzymes were assayed at pH 8.0 with BAEE as substrate.
○-○: soluble; ●-●: CN-EDC-GA

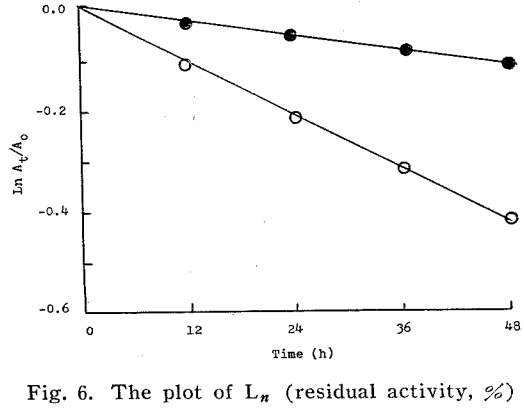

Fig. 6. The plot of L_n (residual activity, %) vs. operation time of immobilized papains at 45°C and pH 8.0 with casein as substrate.
○-○: CN-GA; ●-●: CN-EDC-GA

Table 2. *Operational stabilities of immobilized papain at various temperatures*

The enzyme was batch-operated at pH 8.0 with casein (0.5%) as substrate and 12 h as a batch cycle. Arrhenius equation: $d \log k = (Ea/2.303\ R) \times d\ (1/T)$.

System	Operation temperature (°C)	Denaturation rate const. k (h^{-1})	Fitted Arrhenius equation $y=b+mx$	Ea (Kcal/mol)	Half lives (h)
CN-GA	35	0.0022	$y=16.2-5797x$	26.5	315
	40	0.0052			133
	45	0.0087			80
CN-EDC-GA	45	0.0023	$y=12.5-4811x$	22.0	301
	50	0.0042			165
	55	0.0065			107

× 1/T, where A, R and T represent constant, gas constant and absolute temperature respectively, the activation energy Ea can be calculated and the rate constant at other temperature can be predicted. As shown in Table 1, the denaturation rate constants of immobilized papain during operation at 45°C were 0.0023 h^{-1} and 0.0087 h^{-1} for CN-EDC-GA and CN-GA system, respectively. Therefore, multiple attachment enzyme was 3.8 times more stable than single attachment enzyme at this temperature. From the Arrhenius plot (Fig. 7), it was found that the isokinetic temperature was -6°C and the multiple attachment offered high temperature stability. This is consistent with the theory that multiple attachment on support "freeze" the active enzyme conformation against unfolding. From Arrhenius plots, it was predicted that the operational half lives of CN-GA and CN-EDC-GA system at 20°C were 2786 and 4574 hours, respectively.

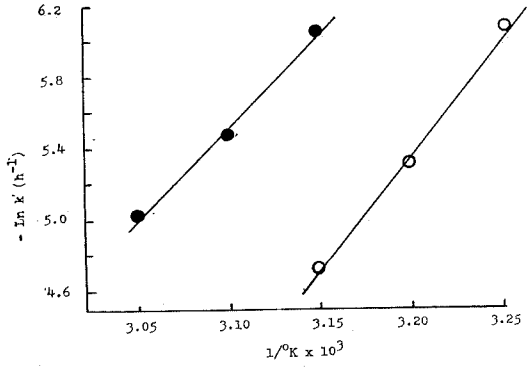

Fig. 7. Arrhenius plots of the denaturation rate constant (k) of immobilized papains in batch-type operation with casein substrate at pH 8.0.
○-○: CN-GA; ●-●: CN-EDC-GA

Table 3. *Thermodynamic parameters for denaturation of immobilized papains during batch operation at 45°C*

System	k (s^{-1})	ΔF^* (Kcal)	ΔH^* (Kcal)	ΔS^* (cal/°K)
CN-GA	2.42×10^{-6}	3.55	25.9	70.3
CN-EDC-GA	6.39×10^{-7}	4.39	21.4	53.5

Considering the denaturation of enzyme as a reaction process, the thermodynamic parameters for the denaturation can be calculated. According to Eyring's transition state theory (Eyring, 1935) $k = k_b\,T/h \times K^*$, where k_b is Boltzmann's constant (1.3807×10^{-23} J · K^{-1}), h is Planck's constant (6.6262×10^{-34} J · S), T is the absolute temperature and K^* the equilibrium constant between activated state and ground state. $\Delta F^* = \Delta H^* - T\Delta S^*$, $k = 0.21 \times 10^{-11}$ S · $K^{-1} \times T \times e^{-\Delta F^*/RT}$, where ΔF^*, ΔH^* and ΔS^* represent the activation free energy, activation enthalpy and activation entropy respectively, R is the gas constant. Assuming ΔS^* is constant within temperature range. $d(\log k)/d(1/T) = -(\Delta H^* + RT)/2.303R$, comparing this with Arrhenius equation $d \log k = -Ea/2.303R \times d(1/T)$, where Ea is the activation energy, we can obtain the following relation ship, $Ea = \Delta H^* + RT$. As shown in Table 3, the CN-GA system was higher than CH-EDC-GA system in both activation enthalpy change(ΔH)* and activation entropy change(ΔS^*) in the denaturation reaction. The positive activation entropy change indicated the increase of randomness in the transition state which was due to the disruption of intramolecular hydrophobic bonding of enzyme (Ohta, 1967). The predominant decrease of ΔS^* (24%) over ΔH^* (17%) accounted for the stability of CN-EDC-GA system over CN-GA system. Since when the enzyme interacts with the support (the formation of external bonds), the bonds within the enzyme molecule are affected, which is accompanied by a decrease of ΔH^*. There is a simultaneous decrease of ΔS^* by covalent fixation of the polypeptide chain to the support. The greater the decrease of ΔS^* (the greater the number of points at which the enzyme in the carrier) compared with ΔH^*, the lower the isokinetic temperature of the immobilized enzymes and the greater the temperature range in which the multiple attachment enzyme is more stable than the single attachment enzyme and the greater the stabilization effect.

Literature Cited

Ax'en, R., J. Porath, and S. Ernback. 1967. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halide. Nature **214**: 1302-1308.

Bass, E. J. and T. Cayle. 1975. Beer. *In* G. Reed (ed.), Enzymes in Food Proccessing. Academic Press, New York, pp 455-471.

Chang, C. T. 1982. Studies on the use of crab chitin as an water insoluble support for the immobilization of pineapple stem bromelain and the preparation of some affinity adsorbants for proteinase. J. Chin. Agri. Chem. Soc. 20: 45-60.

Eyring, H. 1935. Activated complex theory for bimolecular reactions. J. Chem. Phys. 3: 107-119.

Finley, J. W., W. L. Stanley, and C. G. Watters. 1977. Removal of chill haze from beer with papain immobilized on chitin. Biotechnol. Bioeng. 19: 1895-1897.

Fruton, J. S. 1982. Proteinase-catalyzed Synthesis of peptide bonds. *In* A. Meister (ed.), Advances in Enzymology Vol. 53. John Wiley & Sons, Now York, pp. 239-306.

Goldstein, L., Y. Levin, and E. Katchalski. 1964. A water-insoluble polyanionic derivative of trypsin. Biochemistry 3: 1913-1919.

Kilara, A. and K. M. Shahani. 1979. The use of immobilized enzyme in the food industry. CRC critical reviews in food science and nutrition 12: 161-198.

Knorr, D. 1984. Use of chitinous polymer in food. Food Technol. 38: 85-97.

Murachi, T. 1970. Bromelains. *In* S. P. Colowick and N. C. Kaplan, (eds.) Methods in Enzymology, Vol. 19. Academic Press, New York, pp, 273-276.

Ohta, Y. 1967. Studies on the stability of the protease. J. Biol. Chem. 242, 509-515.

Riccardo, A. and A. Muzzarelli. 1980. Immobilization of enzymes on chitin and chitosan. Enzyme Microb. Technol. 2: 177-184.

Srere, P. A. and K. Mosbach. 1974. Metabolic compartmentation. Symbiotic, organellar, multienzymic and microenvironmental. Ann. Rev. Microbiol. 28: 61-83.

多點結合於幾丁聚醣之固定化木瓜酶動力學

蕭介夫 張瑞璋 王盈錦

中央研究院植物研究所

木瓜醣經多點結合法可固定於幾丁聚醣。每克幾丁聚醣能結合之酵素量爲 83.8 mg。固定化木瓜酶對溶性酵素之相對比活性，以酪蛋白爲基質時爲63%，而以 BAEE 爲基質時爲65%。固定化木瓜酶之最適反應 pH 值爲 8.0，比溶性木瓜酶約高1個 pH 單位且其活性對 pH 變化較不敏感。固定化木瓜酶之 pH 穩定性也大爲提高，無論以酪蛋白或 BAEE 爲基質，固定化木瓜酶均比溶性酵素之表觀 K_m 值爲高，而酪蛋白爲基質時其km值之差異較大。多點結合之固定化木瓜酶，其最適反應溫度爲 68°C，比溶性酵素之 52°C 高很多，而其活性也較不易受溫度變化之影響。多點結合酵素（CN-EDC-GA）比傳統之單點結合固定化木瓜酶穩定性大爲提高。以分批式反應操作(45°C)水解酪蛋白時多點結合之木瓜酶變性速率僅爲單點結合酵素之26%。根據 Arrhenius plot 分析此兩種酵素之變性同溫點爲 −6°C，因此多點結合能提供高溫穩定性。在 45°C 下熱變性活化能，活化熵及活化焓，CN-EDC-GA 較 CN-GA 各低17%，24%及17%。