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High-performance affinity chromatography for characterization of human immunoglobulin G digestion with papain

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Abstract

Reactive continuous rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) were prepared within the confines of a stainless steel column. Then papain was immobilized on these monoliths either directly or linked by a spacer arm. In a further step, a protein A affinity column was used for the characterization of the digestion products of human immunoglobulin G (IgG) by papain. The results showed that papain immobilized on the monolithic rod through a spacer arm exhibits higher activity for the digestion of human IgG than that without a spacer arm. The apparent Michaelis–Menten kinetic constants of free and immobilized papain, K_m and V_{max}, were determined. The digestion conditions of human IgG with free and immobilized papain were optimized. Comparison of the thermal stability of free and immobilized papain showed that the immobilized papain exhibited higher thermal stability than the free enzyme. The half-time of immobilized papain reaches about a week under optimum pH and temperature conditions.

Keywords: Monolithic rods; Immunoglobulin G; Papain

1. Introduction

Protein A, a cell wall component produced by *Staphylococcus aureus*, consists of a single polypeptide chain with a molecular mass of 42,000. It has the ability to specifically bind to the Fc region of immunoglobulin molecules, especially IgG [1,2]. Due to the high chemical and thermal stability of the protein, protein A immobilized affinity media have been widely used for the separation of IgGs [3–9]. However, most of the matrices used for the immobilization of protein A are soft gels with poor mechanical strength, thus precluding the use of small particles and high flow-rates to improve resolution and separation time [10]. Alternative supports, so-called monolithic stationary phases, have been developed by Frechet et al. [11,12]. These are cast as a homogeneous column in a single piece and prepared in various dimensions with agglomeration-type or fibrous microstructure. Furthermore, they have many advantages over the traditional chromatographic supports, including relatively easy preparation, unique flow-through and enhanced mass-transfer properties, making them particularly suitable for the separation of biopolymers [13–18]. In our previous work, protein A immobilized monolithic columns

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were prepared and used successfully for the rapid separation of human IgG from serum [19].

The antibody molecule has two separable functions: one is to bind specifically to the pathogen molecules; the other is to recruit cells and molecules to destroy the pathogen. These functions are structurally separated in the antibody molecule [20]. Proteolytic digestion provides a powerful means to determine its structure and functions [21–28]. Antibody molecules can be cleaved into fragments, each of them having a distinct activity. A number of enzymes such as papain, pepsin, bromelain, ficin and elastase have been used for this purpose [24,26], papain being the most frequently used. Most of the enzymatic digestions of antibodies were performed with free enzymes [21–23,25,26]. However, immobilized enzymes offers several advantages over free enzymes, as they allow direct control of the digestion reaction, easy separation of the enzymes from the crude digest, increased stability against denaturation by heat or autolysis. As activity can be maintained for a longer time and the enzyme can be reused, significant savings in enzyme consumption can be achieved [29,30]. Also for the immobilization of enzymes monolithic materials provide some advantageous performance as supports. This has been demonstrated in comparative studies by immobilization of trypsin onto macroporous beads and monoliths [31–33].

In this study, reactive continuous rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) have been prepared by in-situ copolymerization of the monomers in the presence of porogenic diluents within the confines of a stainless-steel tube. Papain was immobilized on these monoliths, in order to be used for the digestion of human IgG. A method for the study of the digestion process was then developed, using protein A affinity chromatography on a monolithic column.

2. Experimental

2.1. Materials

Glycidyl methacrylate (Fluka, Switzerland) and ethylene dimethacrylate (Sigma, MO, USA) were distilled under vacuum. Protein A, human IgG and papain were purchased from Sigma. Azobisisobutyronitrile (AIBN) and dodecyl alcohol were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Cyclohexanol was obtained from Beijing Chemical Reagent Co. (Beijing, China). Human serum was obtained from Dalian maternity hospital. All of the other chemical reagents are analytical grade. Solutions were made in double-distilled water.

2.2. Instruments

A Model 7518-10 peristaltic pump (ColeParmer, USA) and Elite P200 II (Dalian, China) were used to carry out the in situ modification of the monolithic support. A Waters HPLC system consisting of two 515 HPLC pumps, and a 2487 dual wavelength UV detector (Milford, MA, USA), was used to carry out all the chromatographic experiments. The data were acquired and processed with a WDL-97 chromatographic workstation (National Chromatographic R & A Center, Dalian, China).

2.3. Preparation of the monolithic column

The monolithic column was prepared with the procedures reported previously [18]. The porosity and specific area of the monolithic rod were determined according to the procedures previously reported [18].

2.4. Preparation of the papain immobilized column

Fig. 1 shows two approaches adopted for coupling of papain on a poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolith. (1) The column was attached to a peristaltic pump and washed excessively with water. Hydrolysis of the monolith was allowed to proceed for 72 h at a flow-rate of 0.1 ml/min under recirculation of 50 ml HCl solution (pH = 0.5), after which the column was equilibrated with water. After that, 50 ml of 1.5% $NaIO_4$ solution (newly prepared) was pumped through the column to oxidize the medium. Then the 0.1 *M* boracic acid buffer (pH = 8.2) containing 1 mg/ml papain was pumped through the column to react with the aldehyde group at room temperature overnight. Finally, a newly prepared $NaBH_4$ solution was applied to

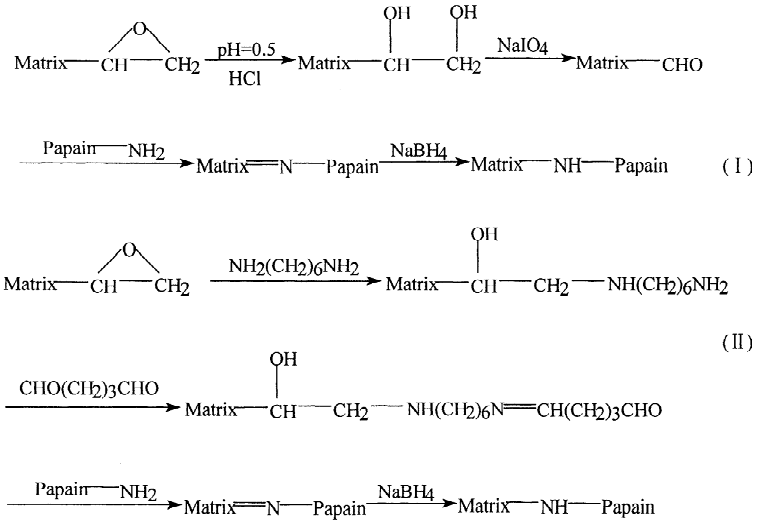

Fig. 1. Schemes for immobilization of papain on poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic rod.

reduce the support overnight. (2) 50-ml solution of excessive hexanediamine in 0.1 *M* sodium bicarbonate, pH = 11.0, was allowed to react with the continuous rod for 4 h at 80 °C. The column was equilibrated with water, and 0.25% glutaric dialdehyde solution was pumped through the column to react with the rod for 4 h at room temperature. Further reaction procedures adopted are the same as the first approach described above. The immobilized papain column was stored in PBS containing 0.1% sodium azide at 4 °C.

2.5. Determination of the amount of papain immobilized on the column

The concentration of papain in solution can be determined by the following empirical equation [3]:

$$C\ (\mathrm{mg/ml}) = 0.144 \times (A_{215} - A_{225}) \qquad (1)$$

where C is the concentration of ligand in solution and A_{215} and A_{225} are the UV absorbance of protein at 215 and 225 nm, respectively. The amount of papain immobilized on the monoliths can be easily calculated from a quantification of the protein concentration in the reaction mixture before and after the immobilization procedure. The amounts of papain immobilized on the poly(GMA-co-EDMA) monoliths through the chemical reactions shown in Eqs. (1) and (2) were determined as 7.1 and 2.9 mg/g support, respectively.

2.6. Preparation of human IgG

A monolithic protein A affinity column was used to purify human IgG from human serum as described previously [19]. The procedures are as follows: human serum was centrifuged at 5000 rev./min for 10 min and then fivefold diluted with loading buffer (20 m*M* phosphate sodium buffer containing 0.15 *M* NaCl, pH = 7.4). The diluted human serum was pumped through the protein A affinity column at a flow-rate of 1.0 ml/min until the column was saturated. Then the column was washed excessively with the loading buffer to remove the non-retained solutes and the excessive human IgG. At last, the elution buffer (0.1 *M* glycine–HCl, pH = 2.3) was used to desorb the human IgG from the column and the human IgG fraction was collected. The purity of human IgG fraction was detected by matrix-assisted laser desorption-ionization time of flight, mass spectrometry (MALDI–TOF–MS), and the results showed that the purity of the obtained human IgG was very high after one-step purification by protein A column [19].

2.7. Production of Fab fragments

Digestion of human IgG to produce Fab fragments with immobilized papain was performed in 75 m*M* sodium phosphate buffer containing 75 m*M* NaCl, 2 m*M* EDTA, and 15 m*M* cysteine. The procedures used in this study are as follows: the human IgG solution obtained from serum was first dialyzed against 75 m*M* sodium phosphate buffer containing 75 m*M* NaCl, 2 m*M* EDTA for 24 h. Then cysteine was added in the solution with a final concentration of 15 m*M*. The papain immobilized rod was pushed out of the column and placed in a test tube. Then the human IgG solution was added to the tube. After incubation at 45 °C, digestion was stopped easily by removing the antibody solution from the tube.

Digestion of human IgG with free papain was performed at 40 °C in the same human IgG solution with a final enzyme:protein ratio of 2% (w/w).

2.8. Production of $F(ab')_2$ fragments

Digestion of human IgG with immobilized papain was performed in 75 m*M* sodium phosphate buffer containing 75 m*M* NaCl, 2 m*M* EDTA to prepare $F(ab')_2$ fragments. The immobilized papain monolith was first activated with 50 m*M* cysteine at 45 °C for 1 h. Then it was washed successively with 75 m*M* sodium phosphate buffer. At last, the human IgG solution without cysteine was added to the papain immobilized monolith for digestion. After incubation at 45 °C, digestion was stopped easily by pouring the antibodies solution out of the tube.

2.9. Study of the digestion process by protein A affinity column

Protein A column was first equilibrated with loading buffer (20 m*M* phosphate sodium buffer containing 0.15 *M* NaCl, pH = 7.4). Then the digested antibody mixture was loaded on the protein A column and the non-retained Fab or $F(ab)_2$ fragments were washed out of the column with loading buffer (peak 1). Non-digested IgG and Fc fragments were eluted from the column by successive washing with 0.1 *M* glycine–HCl buffer, pH = 2.3 (peak 2). The area of peak 1 increased but the area of peak 2 decreased while digestion proceeded. The area of peak 2 is directly correlated to the quantity of IgG cleaved. Therefore, the area of peak 2 could be used as a criterion for the evaluation of the digestion effectiveness.

2.10. Analysis of digestion products by HPLC–GPC

The digestion mixtures were analyzed by high-performance size-exclusion chromatography (HP-SEC). Separation was achieved on a 7.8 mm × 30 cm column packed with Spherogel TSK 3000 SW with a 7.8 mm × 10 cm Spherogel TSK SW precolumn installed. Separation was performed isocratically with 0.1 *M* phosphate sodium buffer, pH 7.0 at a flow-rate of 0.3 ml/min and monitored at 280 nm.

3. Results and discussion

Monolithic stationary phases can be tailored to have extremely large pores, which then provide channels with very low flow resistance. Therefore, rapid and efficient separation of biomolecules can be obtained within a very short time. A representative chromatogram of standard human IgG solution on a protein A monolithic column is shown in Fig. 2a. It can be seen that an immunoaffinity analysis could be

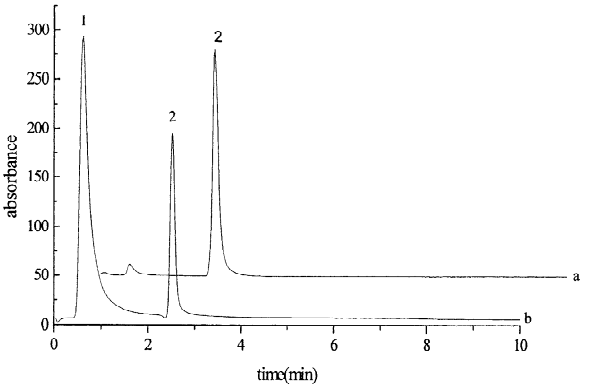

Fig. 2. Chromatograms of human IgG digestion products in the presence of cysteine on protein A monolithic column. Conditions: column, 50 × 4 mm I.D.; 20 μl of the digestion product were injected with loading buffer, and 2 min later the elution buffer was used as the eluent. Flow rate, 1.0 ml/min; UV detection at 280 nm. (a) Human IgG solution prior to digestion, (b) human IgG digestion product. Peaks: 1, Fab fragment and impurities; 2, human IgG and Fc fragment.

finished within 3 min at a flow-rate of 1.0 ml/min, and the peak width at half height for human IgG is only 0.1 min. Due to the high specific affinity of protein A towards the Fc region of immunoglobulins, immobilized protein A media can be used to remove the undigested IgG and Fc fragments from the digestion solution, and therefore pure Fab or $F(ab')_2$ fragments can be easily obtained [27]. Digestion into these fragments can be performed by incubation of the antibody with papain. The latter is a non-specific, thiol-endopeptidase containing a sulfhydryl group at the active site, which must be in the reduced form for activity [34]. However, if no reducing agent is presented during IgG digestion, two fragments including one $F(ab')_2$ fragment and one Fc fragment, will be generated. In our work, a protein A monolithic column was used to investigate the digestion process of human IgG with papain. Fig. 2b shows the chromatograms of IgG digestion product resulting from the digestion by papain in the presence of cysteine on the protein A monolithic column. There, peak 1 represents the Fab fragments and peak 2 represents the Fc fragments together with IgG. A chromatogram of an IgG digestion by papain in the absence of cysteine on the protein A monolithic column is shown in Fig. 3. There, peak 1 represents the $F(ab')_2$ fragments and peak 2 represents the Fc fragments together with IgG. From Figs. 2 and 3, it can be seen that the area of peak 1 increased while the area of peak 2 decreased as the digestion proceeded. The area of peak 2 is directly correlated to the quantity of the human IgG cleaved. Therefore, the area of peak 2 could be used as a criterion for the evaluation of the digestion effectiveness.

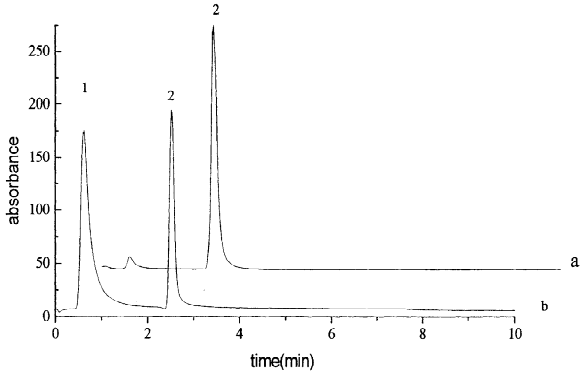

Fig. 3. Chromatograms of human IgG digestion products in the absence of cysteine on protein A monolithic column. (a) Human IgG solution prior to digestion, (b) human IgG digestion products. Peaks: 1, $(Fab')_2$ fragment and impurities; 2, human IgG and Fc fragment. Other conditions are the same as in Fig. 2.

Prior to digestion, peak 2 represents only human IgG molecules

$$A_0 = E_{280}^{\mathrm{HIgG}} C_1 \tag{2}$$

When digestion proceeds, peak 2 represents Fc fragments and undigested human IgG

$$A_1 = E_{280}^{\mathrm{HIgG}} C_2 + E_{280}^{\mathrm{Fc}} (C_1 - C_2) \tag{3}$$

When digestion is complete, peak 2 represents only Fc fragments

$$A_2 = E_{280}^{\mathrm{Fc}} C_3 \tag{4}$$

where, A represents the UV absorbance; E_{280}^{HIgG} and E_{280}^{Fc} represent the molar absorption coefficients of human IgG and Fc fragments at 280 nm, respectively; C_1 represents the initial molar concentration of human IgG; C_2 represents the molar concentration of human IgG when digestion proceeds; C_3 represents the final molar concentration of Fc fragments after completed digestion. Because 1 mole human IgG produces 1 mole Fc fragments, the final molar concentration of Fc fragments equals the initial molar concentration of human IgG ($C_1 = C_3$). The molar absorption coefficients of human IgG and Fc fragments could be easily obtained by determining the absorbance prior to and after the digestion is complete. When E_{280}^{HIgG} and E_{280}^{Fc} are replaced in Eq. (3), C_2 at any time could be easily determined. Because the height of peak 2 is directly correlated to the absorbance, C_2 at any time could be determined by measuring the height of peak 2.

The activity of the immobilized papain for human IgG in the presence of cysteine is 17% of the free papain as determined by the method described above. The loss in activity is suspected to be due to the immobilization process, but also due to steric hindrance during the digestion.

The efficacy of an immobilized enzyme is governed by the properties of the enzyme itself, but also by the properties of the carrier material [29,31–33], such as shape, porosity, chemical stability, and mechanical strength. According to mercury intrusion

porosimetry, the monolithic material used in this study has an average pore size of 769 nm and specific surface area of 89.1 m^2/g. The total pore volume reaches to 3.3 ml/g with a porosity over 75%.

As IgG is a rather large molecule, steric hindrance can play an important role during the interaction with the immobilized ligand. This phenomenon will cause a reduction or complete lack of specific binding [1,3]. In order to overcome this problem, a spacer arm was bound to the support prior to attachment of the ligand. Thereafter, the digestion effectiveness of papain immobilized on the monoliths either by the reaction described by Eq. (I) or by that described by Eq. (II) was investigated. The two-step modification process shown in Eq. (II) involving reaction with hexanediamine followed by the reaction with glutaric dialdehyde introduced a spacer arm of a length of 11 carbon atoms prior to immobilization of papain on the monolithic carrier. The introduction of this spacer arm led to significant improvement of the digestion effectiveness. When papain was immobilized directly on the monolith the latter was only 56% of that of papain immobilized through the spacer arm.

Digestion of IgG with immobilized papain in the presence of 0.01 *M* cysteine led to an efficient cleavage of IgG into Fab and Fc fragments after incubation for 8 h at 45 °C. The chromatogram of the digestion on Spherogel TSK 3000 SW column is shown in Fig. 4a. Two main peaks can be distinguished besides some partially digested material. The first main peak represents the undigested IgG while the second represents Fc and Fab fragments. In the absence of cysteine, IgG was only partially cleaved by the immobilized papain. The chromatogram of the digestion products separated on Spherogel TSK 3000 SW column is shown in Fig. 4b. There are three partially separated main peaks. They represent IgG, $F(ab')_2$ fragments, and Fc fragments, respectively.

Hanes plots of $[S]/\nu$ versus $[S]$, a linearized form of the Michaelis–Menten equation, can be used to express enzymatic digestions:

$$\frac{[S]}{\nu} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} \cdot [S] \quad (5)$$

where $[S]$ is the substrate concentration (mg/ml); ν the velocity of enzymatic reaction (mg/ml min^{-1}); V_{max} the maximum velocity of enzymatic reaction (mg/ml min^{-1}); K_m the apparent Michaelis–Menten kinetics constant (mg/ml). The experimental data processed by the Hanes plots are shown in Fig. 5. The apparent K_m of the immobilized papain for human IgG is 0.55 mg/ml, which is about 50% of that of free papain (1.08 mg/ml). The decrease in this value for immobilized enzymes has been widely recognized, and has been ascribed to the increased substrate concentration around the polymer due to

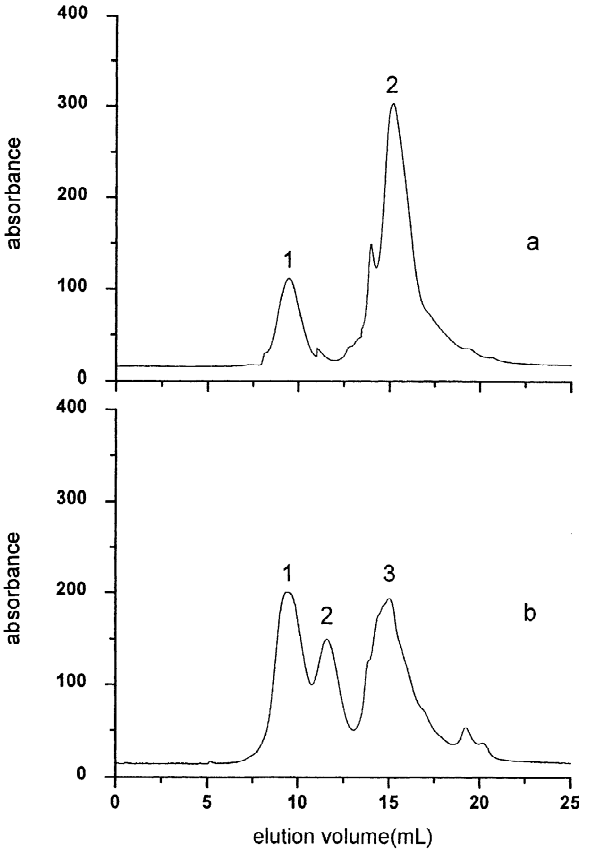

Fig. 4. Chromatograms of human IgG digestion products on Spherogel TSK 3000 SW column. Conditions: column, 300×7.8 mm I.D. with a 100×7.8 mm I.D. precolumn installed; mobile phase, 0.1 mol/l phosphate sodium buffer, pH 7.0; flow-rate, 0.3 ml/min; UV detection at 280 nm. (a) Digestion in the presence of cysteine. Peaks: 1, human IgG; 2, Fab and Fc fragments. (b) Digestion in the absence of cysteine. Peaks: 1, human IgG; 2, $(Fab')_2$ fragments; 3, Fc fragments.

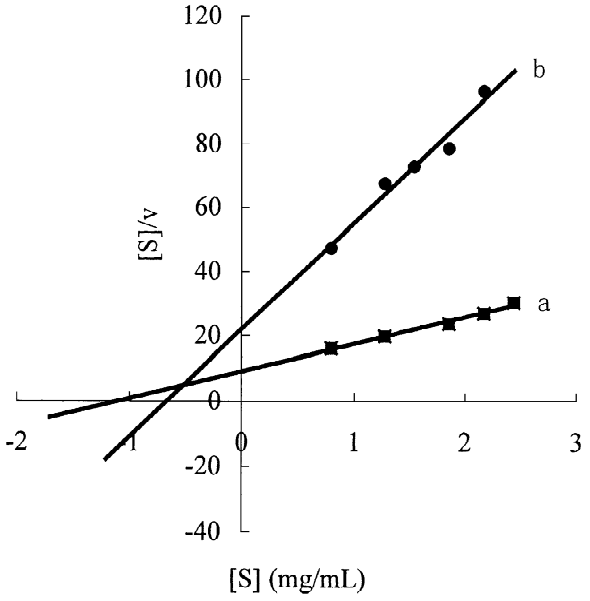

Fig. 5. Hanes plots for (a) free and (b) immobilized papain.

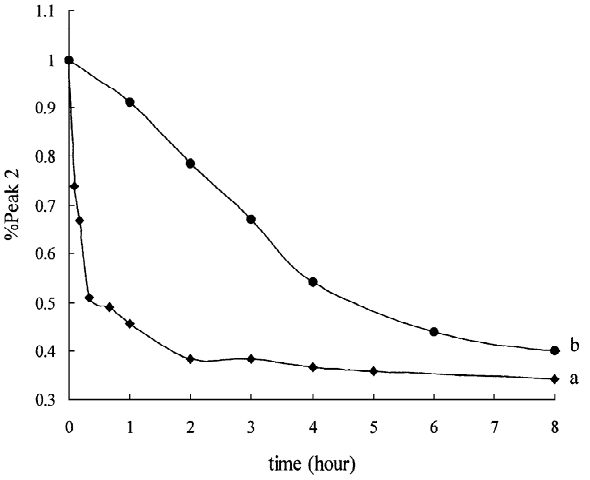

Fig. 6. Effect of incubation time on the digestion effectiveness of (a) free and (b) immobilized papain.

electrostatic attraction and hydrophobic adsorption of the substrate on the solid materials [30,35]. The maximum velocity of free and immobilized papain for human IgG is 0.12 and 0.03 mg/ml min^{-1}, respectively.

Digestion conditions, such as incubation time, pH, and temperature, have a significant effect on fragment yield. Immobilization of enzyme involves deliberate restriction of the mobility of the enzyme, which in turn can also affect the mobility of the substrates. These phenomena, referred to as mass transfer effects, can lead to a reduced reaction rate and consequently to a decreased efficiency compared with free enzymes. Fig. 6 shows the effect of incubation time on Fab fragment yield from human IgG with free and immobilized papain. The data points are calculated from the area of peak 2 (IgG and Fc fragments as described above, see Figs. 2 and 3), which is directly correlated to the quantity of the cleaved IgG. It can be seen that a 2-h incubation and an 8-h incubation are optimal for cleavage of IgG with free and immobilized papain, respectively. Therefore the optimal incubation time for cleavage of IgG with immobilized papain is much longer than that with free papain, which was consistent with the result obtained by Coulter et al. [27]. This phenomenon may be explained by the restricted mass transfer, as developed by Tischer and Kasche [29].

The pH effect on the rates of enzyme reactions can be explained by the occurrence of three forms of the enzyme shown as follows [36]:

$$EH_2 \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} EH \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} E$$

At the optimum pH value the concentration of the intermediate form reaches the maximum. When the pH is changed, this form will be transformed into other forms. The effect of pH on the digestion effectiveness of free and immobilized papain for human IgG was investigated and it was observed that the optimal digestion proceeds at pH values of 7.0 and 7.5. The optimum pH for immobilized papain is 0.5 units higher than that of free papain.

The effect of temperature on the rates of enzyme reaction is somewhat complicated. Raising the temperature affects two independent processes, the catalyzed reaction itself and the thermal inactivation of the enzyme. In the lower temperature range, inactivation is very slow and has no appreciable effect on the rate of the reaction; the overall rate therefore increases with increasing temperature. At higher temperature however, inactivation becomes more and more important, so that the concentration of active

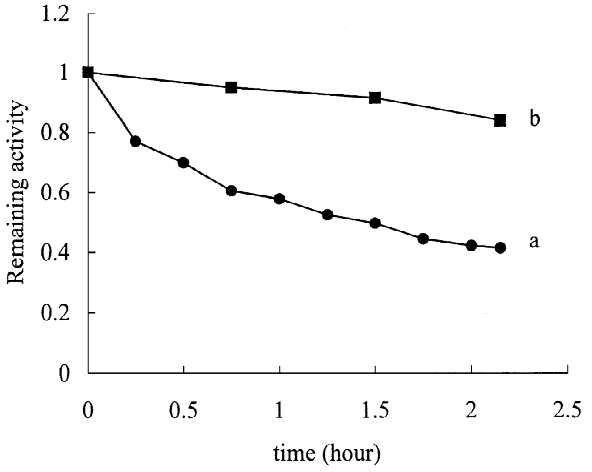

Fig. 7. Thermal stability of (a) free and (b) immobilized papain at 75 °C.

enzyme decreases during the course of reaction [36]. It was observed that the optimum temperature for immobilized enzyme is 5 °C higher than that for free papain, which may be explained by the increased stability of the immobilized enzyme against heat denaturation.

Consequently, free and immobilized papain were incubated at 75 °C for 2 h to investigate their thermal stability. The remaining activity was determined at intervals by measuring the effectiveness for the digestion of human IgG. The obtained result is shown in Fig. 7. It can be seen that free papain lost its activity by 60% whereas immobilized papain only lost 10% of its activity during this incubation procedure. Thus the thermal stability of papain was greatly improved after being immobilized on the poly(GMA-co-EDMA) monolith. The operational stability (usually expressed as catalytic half-life $\tau_{1/2}$) is another important parameter for the characterization and evaluation of immobilized enzyme. The catalytic half-life of the immobilized papain reached about 1 week under optimum pH and temperature conditions.

4. Conclusion

Reactive poly(glycidyl methacrylate-co-ethylene dimethacrylate) monolithic rods were used for the immobilization of papain. The effectiveness of these rods for the digestion of IgG with papain was investigated by analyzing the digestion products on a second monolithic affinity column, where protein A had been immobilized. By this means, the apparent Michaelis–Menten kinetic constants K_m and V_{max} of free and immobilized papain for human IgG could be compared and the digestion conditions for both were optimized. It was observed that the K_m of the immobilized papain only reached about 50% of that of free papain. However, the thermal stability of the immobilized papain was much higher than that of free papain and the enzymatic half-time of the immobilized papain reached about 1 week under the optimum pH and temperature.

[36]

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