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Casein hydrolysis by immobilized enzymes in a torus reactor

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Abstract

A torus reactor was used to perform hydrolysis of casein with a protease immobilized on beads. Chitosan beads with two different sizes were tested as immobilization matrices. The mechanical resistance of beads in the torus reactor was tested for different times and rotation speeds. Chitosan beads were considered to be suitable as an immobilizing support. The more stable hydrolysis rates in successive assays were obtained with the protease grafted onto the largest chitosan beads. The kinetics parameters of free and immobilized enzymes were determined and compared in batch stirred tanks and torus reactors. Immobilization decreased the enzyme activity to only 5% of the free enzyme activity. No significant differences for apparent kinetic constant (K_m) were observed between immobilized and free enzyme. The apparent reaction rate constant (V_{max}) was 25 times less with immobilized enzyme in the torus reactor than with free enzyme in the batch reactor.

Keywords: Enzymic hydrolysis; Torus reactor; Immobilized enzyme; Casein; Kinetics

1. Introduction

Polysaccharides and proteins are produced by agricultural, animal and marine industries. As they present high biological, technological and economical interest, it is possible to enhance their use in different food and non-food applications by diversifying and improving their functional properties by enzymic processes.

In order to improve enzymic processes economically, the enzyme could be used in continuous processes over a long time period in order to exploit it completely. Another possibility is to try to recover the enzyme and stop the reaction without drastic treatments which could alter its activity and possible reuse. In contrast to these processes, immobilized enzyme processes offer the following benefits: stability, repetitive uses, possibility to stop the reaction easily and obtain product which cannot be contaminated by the enzymes.

The purpose of this work was a preliminary study of casein hydrolysis with immobilized enzymes in a torus reactor. A torus reactor, which can be considered as a loop reactor, presents some advantages over other stirred tank reactors including efficient mixing of reactants [1,2], easy scale-up and design ensured by the absence of dead volume, low power consumption [3], high heat transfer capacity [4], prevention of deposition of polymer or biomaterial on the reactor wall [4,5] and efficiency [3,6,7].

Previous studies with a torus reactor for food protein transformation illustrated these advantages in the acetylation of pea protein isolate [6] and for enzymic hydrolysis of wheat proteins [3]. The reactor gave better conversion rates for given energetic criteria than a stirred tank reactor. Finally, as consequence of its characteristics, the torus reactor will be able to ensure circulation of enzymes immobilized onto particles.

The main objective was to examine the feasibility of the hydrolysis of casein in a torus reactor. Casein was chosen as a model because its hydrolysis has been widely studied and well known in the cheese ripening industry [8–12]. The

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suitability of chitosan as a support for immobilizing enzyme was investigated and kinetics parameters of the hydrolysis were determined. Long time batch runs with free enzyme and continuous runs with immobilized enzyme are also presented.

2. Materials and methods

2.1. Protein and protease

Casein was purchased from Merck. This casein contains 90% protein and was used without further purification. For this study, casein was solubilized in sodium phosphate buffer (0.05 M, pH 7). The enzyme used in the study was the protease XIX from *Aspergillus sojae*, purchased from Sigma–Aldrich (ref. P7026, CAS Number: 9001-92-7, approx. 0.4 U/mg solid).

2.2. Enzyme immobilization on beads

Chitosan beads, supplied by E. Guibal (laboratoire de Génie de l'Environnement Industriel, Ecole des Mines d'Alès, France), were prepared by an original procedure [13]. The five batches differed by the bead diameter and the chitosan percentage. In addition, two of these batches were crosslinked with glutaraldehyde before immobilization in order to enhance the rigidity of the beads (Table 1). Protease XIX was covalently immobilized with glutaraldehyde on chitosan beads by a procedure adapted from Carrara and Rubiolo [14]. Four hundred microlitres of glutaraldehyde solution (25%, v/v) was added to 5 g of chitosan beads in 10 ml of sodium phosphate buffer (0.05 M, pH 7) at room temperature and maintained with periodic stirring for 4 h. The beads were then washed three times with phosphate buffer. The last washing solution was analyzed by spectrophotometry (280 nm) to control the complete elimination of glutaraldehyde. Ten millilitres of protease XIX (50 mg/ml) was added on the beads and maintained at 8 °C with rotative stirring during 12 h. The beads were washed again three times with phosphate buffer. The last washing solution was also analyzed by spectrophotometry (280 nm) to control the elimination of free enzyme. Beads were stored in phosphate buffer at 4 °C. Values for enzyme loading are presented in Table 1. The best value of immobilization was obtained with batch no. 1 characterized by the highest

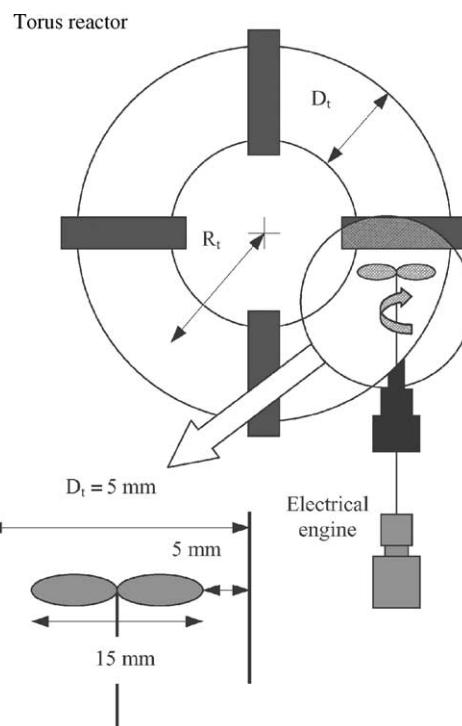


Fig. 1. Sketch of torus reactor.

diameter. The values achieved for crosslinked beads were lower, the action of glutaraldehyde during the procedure of reticulation could have modified the structure of the beads.

2.3. Stirred tank and torus reactors

The stirred tanks were a series of 10 ml glass tubes fixed on a rotating horizontal axis connected to an electrical motor. This provided a gentle stirring of 5 rpm.

The torus reactor used in this study (Fig. 1) was made of transparent Plexiglas with a planar surface and was disposed horizontally. It had a volume of 100 ml, an average length (L_t) of 160 mm and an internal diameter (D_t) of 25 mm. The agitation system was constituted by a marine propeller with three Teflon blades with external diameter (d_1) of 15 mm, internal diameter (d_2) of 5 mm, a blade angle (ϕ) of 22.5°. The gap between the blades and the inner wall of the reactor was 5 mm. The propeller was connected to an electrical motor (IKA-WERK, RW 20 DZM). The reactor was placed in an incubator to achieve experiments at controlled temperatures.

Table 1
Characteristics of the chitosan beads

| Batch no. | Diameter (D_p in mm) | Chitosan (%) | Reticulation | Enzyme Immobilized (mg enzyme) | Percentage of enzyme immobilized |
|-----------|-------------------------|--------------|--------------|--------------------------------|----------------------------------|
| 1 | 2.76 | 3.5 | No | 464 | 92.8 |
| 2 | 3.39 | 7.0 | No | 432 | 86.4 |
| 3 | 3.45 | 7.0 | Yes | 374 | 74.8 |
| 4 | 1.03 | 3.5 | No | 415 | 83.0 |
| 5 | 1.03 | 3.5 | Yes | 360 | 72.0 |

2.4. Determination of degree of hydrolysis by measurement of free-amino groups

The degree of hydrolysis (DH) or conversion was determined following the estimation of NH₂ groups. The NH₂ groups were determined by the OPA (*o*-Phthaldehyde) method [15]. Amino groups react with *N,N*-dimethyl-2-mercaptoethyl ammonium chloride (DMMAC) to give an isoindol which was detected by spectrophotometry (340 nm). A calibration curve was established with known concentration of L-leucine. The degree of hydrolysis, the percentage of peptic bonds broken during hydrolysis can be determined by the relationship:

$$\text{DH (\%)} = \frac{h}{h_0} \times 100 \quad (1)$$

where h is the quantity per gram of amino groups liberated during hydrolysis and h_0 the total quantity of amino groups liberated during a total hydrolysis of one gram of protein. h was directly determined on each hydrolysate sample and h_0 was determined after total hydrolysis of the protein by 6N hydrochloric acid at 110 °C during 24 h under vacuum as described in literature [15].

3. Results and discussion

3.1. Mechanical resistance of beads in the torus reactor

Tests were carried out with chitosan beads to determine their resistance during hydrolysis experiments. The determination of mechanical deformation was achieved by direct visualization with a video camera and by using OPTIMAS software for image analysis in order to determine the number, and contour of the beads and their diameter. After a first visualization, the beads were introduced into the torus reactor with 100 ml of sodium phosphate buffer. Several runs at different rotation speeds and residence times were achieved. After each run, a second visualization allowed the determination of the mechanical resistance of the beads in the torus reactor by counting the number of intact beads.

Tests were carried out with large and small chitosan beads (Table 1). Samples were constituted of approximately 100 beads for the large ones and 1100 for the small ones, suspended in 100 ml of sodium phosphate buffer (0.05 M, pH 7.0). Between two consecutive runs of 360 min, beads were stored in buffer at 4 °C for several days. The detailed operating conditions for the two types of beads are reported in Table 2.

The mechanical study showed that globally the small beads batches no. 4 and 5 had an excellent mechanical resistance (Table 2). During a 360 min experiment, less than 0.5% of the beads were destroyed, although a relatively high speed was required to move the beads. No effect of crosslinking was noted.

Beads of batch no. 1 (large beads) had also a good mechanical resistance, although their diameter was not much smaller than the gap between the reactor wall and the propeller blades. For the other large beads, surface erosion was observed during the first experiment and formation of small particles during the second. Between 20 and 45% of the beads were destroyed. This could be due to a higher brittleness of the material, resulting from the higher chitosan concentration or/and the crosslinking that make the beads more sensitive to contact with the propeller blades. Therefore, beads from batches no. 1 and 4 have been chosen for hydrolysis reactions. Beads with crosslinking were not used for hydrolysis experiments, because of both bad mechanical resistance and low ratio of immobilized enzyme that made these beads less attractive than the other ones.

3.2. Hydrolysis experiments

This preliminary study was not designed to determine optimal temperature and pH conditions. Nevertheless, attention was paid to the choice of pH and temperature. As related, it is appropriate to work approximately under physiological conditions, pH 7.5 and 37 °C [16]. Experiments at pH 7 were appropriate for good solubilization of casein.

Preliminary casein hydrolysis assays were performed with free and immobilized enzyme in the 10 ml stirred reactor. Conditions were as follows, casein concentration 5 mg/ml in 5 ml of sodium phosphate buffer (0.05 M, pH 7), free

Table 2
Resistance of the chitosan beads in the torus reactor

| Batch no. | No. consecutive assays | Beads initial number | Min. stirring speed (rev/min) | Stirring speed (rev/min) | Time (min) | Beads final number | Aspect | |
|-----------|------------------------|----------------------|--|--------------------------|------------|--------------------|--------------------|--|
| 1 | 1 | 100 | 1200 | 1600 | 360 | 100 | Intact | |
| 1 | 2 | 98 | 1200 | 1600 | 360 | 98 | Intact | |
| 2 | 1 | 107 | 1500 | 1800 | 360 | 107 | Surface erosion | |
| 2 | 2 | | 90 + small bits, bad conditions, weakening during run or storage | | | | | |
| 3 | 1 | 70 | 1000 | 1500 | 360 | 67 | Intact, small bits | |
| 3 | 2 | | 57 + small bits, bad conditions, weakening during run or storage | | | | | |
| 4 | 1 | 1143 | 1000 | 1800 | 360 | 1142 | Intact | |
| 4 | 2 | 1141 | 1000 | 1800 | 360 | 1138 | Intact | |
| 5 | 1 | 1129 | 1700 | 1800 | 360 | 1126 | Intact | |
| 5 | 2 | 1125 | 1700 | 1800 | 360 | 1123 | Intact | |

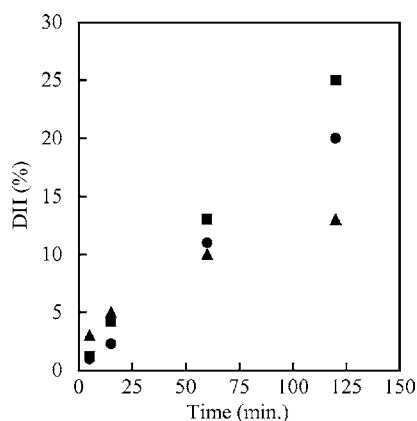


Fig. 2. Casein hydrolysis for beads of batches no. 1 and 4 and free XIX protease in stirred reactor ((●) beads of batch no. 1; (■) beads of batch no. 4; (▲) free enzyme).

enzyme (1/62.5 enzyme–substrate ratio, 0.4 mg of enzyme) or immobilized enzyme (250 mg of beads, 23.20 mg of immobilized enzyme for batch no. 1 and 20.75 mg for batch no. 4, see Table 1) and temperature of 37 °C. Reaction times were 5, 15, 60 and 120 min.

The reaction in the 10 ml reactor (Fig. 2) showed that it is possible to reach high degrees of hydrolysis (higher than 20%) with the protease immobilized on small as well as large beads. The reaction was approximately linear over 2 h, whereas that of hydrolysis with the free protease tended towards a plateau, but the quantity of enzyme immobilized was 50 times higher with the immobilized protease (23.20 mg for batch no. 1, 20.75 mg for batch no. 4) than used with the free protease (0.40 mg). Thus, the relative activity of immobilized enzyme is estimated at about 3% of free enzyme activity for both bead batches. The decrease of enzymic activity after immobilization has been also observed with β -galactosidase immobilized by the same method on chitosan beads [14], and can be explained by enzyme denaturation resulting from glutaraldehyde action. In the present case, this loss of activity of immobilized enzyme with respect to free enzyme could be counterbalanced by the reuse of the enzyme (20–30 times). Lilly and Dunnill [17] have shown that immobilized enzymes could be reused 50 times without decrease of hydrolysis rates. However the main interest of immobilized proteases is to make it easier to stop the reaction and to improve the recovery of functional products.

Consecutive runs of casein hydrolysis with immobilized enzyme were also performed in the stirred reactor (10 ml) and in the torus reactor (100 ml), this last with a moderate agitation (1300 rpm). The operating conditions were: casein concentration of 5 mg/ml in 5 ml (stirred) or 100 ml (torus) of sodium phosphate buffer (0.05 M, pH = 7), 1/10 immobilized enzyme–substrate ratio, 25 mg of immobilized beads (batches no. 1 and 4, see Table 1) in stirred reactor or 500 mg in torus reactor, 37 °C and reaction time of 5, 15, 60 and 120 min.

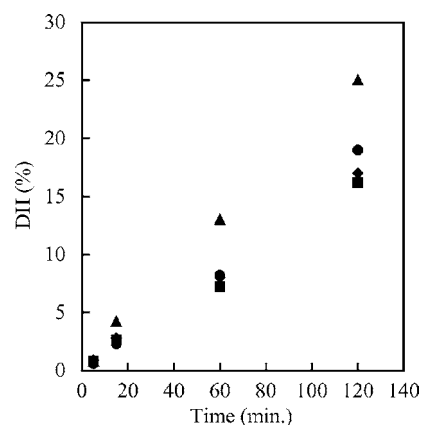


Fig. 3. Hydrolysis conversion in consecutive runs for beads of batches no. 1 and 4 immobilized XIX protease in stirred reactor ((◆) beads of batch no. 1, first run; (■) beads of batch no. 1, second run; (▲) beads of batch no. 4, first run; (●) beads of batch no. 4, second run).

In the stirred reactor, the small beads (batch no. 4) yielded higher degrees of hydrolysis than the large one (batch no. 1) in the first run (Fig. 3). During the second run the loss of activity of small beads after 120 min was high and the final DH was 74% of the first run DH. Conversely, the activity of the large beads decreased only slightly (second run DH = 95% of the first run DH).

Experiments carried out in the torus reactor confirmed that the activity of the large beads remained almost constant during the process, DH at the end of the second run of 120 min being 94% of DH at the end of the first run (Fig. 4). With the small beads, the hydrolysis rate reached at the end of the second run was only 79% of that obtained at the end of the first run.

These results confirmed the feasibility of hydrolysis in the torus reactor of a food protein with a protease immobilized on chitosan beads. Considering that the stability of the activity is a key requirement of the immobilized enzymes for the development of a reproducible procedure, chitosan large beads of batch no. 1 were chosen for further kinetic studies.

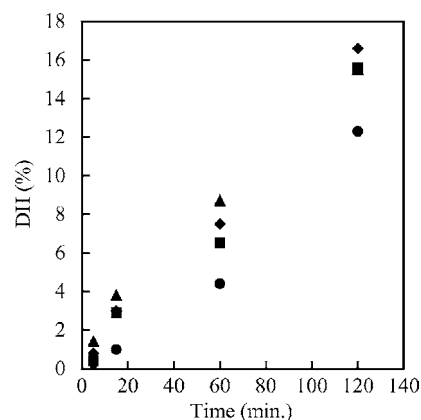


Fig. 4. Hydrolysis conversion in consecutive runs for beads of batches no. 1 and 4 immobilized XIX protease in torus reactor ((◆) beads of batch no. 1, first run; (■) beads of batch no. 1, second run; (▲) beads of batch no. 4, first run; (●) beads of batch no. 4, second run).

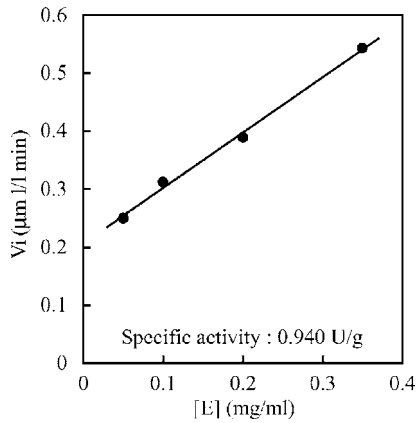


Fig. 5. Activity of free XIX protease in casein hydrolysis with stirred reactor.

3.3. Free and immobilized enzyme activities

Free and immobilized protease activities were determined in stirred reactor with sodium phosphate buffer (0.05 M, pH 7) at 37 °C, with 5 mg/ml casein and 0.05, 0.10, 0.20 and 0.35 mg/ml free enzyme or 20, 40, 70 and 100 mg/ml immobilized enzyme beads of batch no.1 (Table 1).

Hydrolysis assays were carried out for 30 min to allow a sufficient rate of hydrolysis for each concentration and enable a more precise measure of quantities of reacted substrate to be produced. These quantities are close to 1% total substrate quantity that could be hydrolyzed in order to work in initial hydrolysis conditions and permit the assumption of a linear time-evolution of the degree of hydrolysis [16]. The enzyme activity can be determined by the slope of the apparent reaction rate versus enzyme concentration. Enzymic activities of free and immobilized protease are presented in Figs. 5 and 6. The activity of the free enzyme was equal to 0.940 $\mu\text{mol}/(\text{min mg enzyme})$ or 0.940 U/g (where one unit (U) of hydrolytic activity is 1 μmol peptide bond cleaved per minute), while that of the immobilized enzyme was 0.042 $\mu\text{mol}/(\text{min mg enzyme})$ or 0.042 U/g, considering that

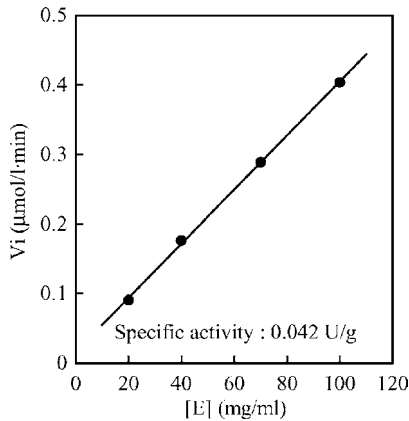


Fig. 6. Activity of immobilized XIX protease on chitosan big beads of batch no. 1 in casein hydrolysis with stirred reactor.

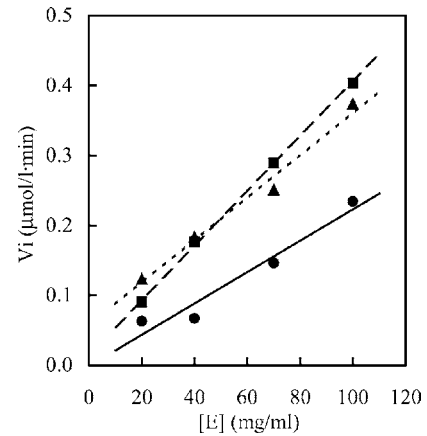


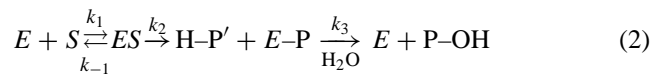
Fig. 7. Activity of immobilized XIX protease on chitosan big beads of batch no. 1 in casein hydrolysis with stirred reactor, effect of temperature ((●) $T = 25^\circ\text{C}$; (■) $T = 37^\circ\text{C}$; (▲) $T = 44^\circ\text{C}$).

92.8 mg of enzyme was immobilized on 1000 mg of beads. This represents only 4.5% free enzyme activity and is in agreement with the value estimated from the degrees of hydrolysis reported above.

The free enzyme has its optimal activity temperature at 37 °C. After immobilization behavioural changes occurred and it is necessary to verify if this temperature is optimal. For example, some differences have been observed with β -galactosidase, working better at 44 °C after immobilization than at 37 °C [14]. Fig. 7 shows that the apparent reaction rate at 25 °C is slower than at 37 or 44 °C. There are no difference between 37 and 44 °C, the performance of enzyme was quite similar. The life time of enzyme at 44 °C should be lower than that at 37 °C [14], then the optimal temperature is equal to 37 °C.

3.4. Kinetics parameters

The hydrolysis kinetics of reaction studied can be expressed by the following equation:



where k_1 , k_{-1} , k_2 and k_3 are the rate constants. The apparent reaction rate (V_i) is given by the Eq. (3) derived from the Michaelis model [18]:

$$V_i = V_{\max} \frac{[S]}{[S] + K_m} \quad (3)$$

where V_{\max} is the maximum apparent reaction rate and K_m the apparent kinetic constant. K_m is expressed by the Eq. (4):

$$K_m = \frac{k_{-1} + k_2}{k_1} \frac{k_3}{k_2 + k_3} \quad (4)$$

and the apparent catalytic constant k_{cat} by (5):

$$k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_3} \quad (5)$$

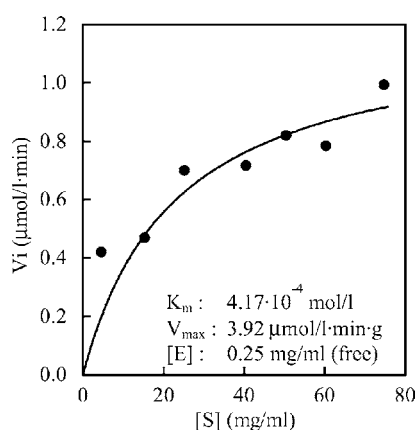


Fig. 8. Kinetics of free XIX protease on chitosan big beads of batch no. 1 in casein hydrolysis, stirred reactor, evolution of V_i ($[S]$) and determination of V_{max} and K_m (●● experimental values; (—) non-linear regression).

In the case of immobilized enzymes when no porous support is used (no intraparticle diffusion phenomena), extraparticle diffusion phenomena and mass transfer resistance can be neglected. Then, K_m values for immobilized and free enzymes are similar [19].

Several graphic representations allows determinations from the Michaelis–Menten model (Eq. (3)), of the values of K_m and V_{max} [20]. In this study experimental values were fitted to the curve given by Eq. (2). A non-linear regression was used to determine values of K_m and V_{max} .

Hydrolysis assays achieved over 30 min had a sufficient hydrolysis rate for each concentration allowing a more precise measure of reacted substrate quantities. The apparent reaction rate (V_i) was determined, after titration of samples, by calculating the number of substrate moles reacted by litre of solution and per minute. The kinetics studies were carried with the hydrolyses of casein by protease XIX, free or immobilized on chitosan beads.

Experimental conditions for free enzyme in the stirred reactor were casein concentration of 5, 15, 25, 40, 50, 60 and 75 mg/ml, 5 ml sodium phosphate buffer (0.05 M, pH 7.0) at 37 °C and 0.25 mg/ml free enzyme. Experimental conditions for immobilized enzyme in the stirred reactor were casein concentration of 1, 3, 5, 7, 10, 13, 16 and 20 mg/ml, 5 ml sodium phosphate buffer (0.05 M, pH 7.0) at 37 °C and 50 mg/ml immobilized enzyme beads (batch no. 1, see Table 1). Experimental conditions for the immobilized enzyme in the torus reactor were casein concentration of 1, 3, 5, 7, 10, 15 and 20 mg/ml, 100 ml sodium phosphate buffer (0.05 M, pH = 7) at 37 °C, 25 mg/ml immobilized enzyme beads (batch 1, see Table 1) and agitation of 1300 rpm.

Experimental curves allowing the determination of kinetic parameters of the free and immobilized enzyme in the stirred and torus reactors are given in Figs. 8–10. The plot of V_i versus $[S]$ is a rectangular hyperbola in all cases, showing a Michaelian behaviour [16]. The values of K_m and V_{max} derived from the curves are reported in Table 3. As can be

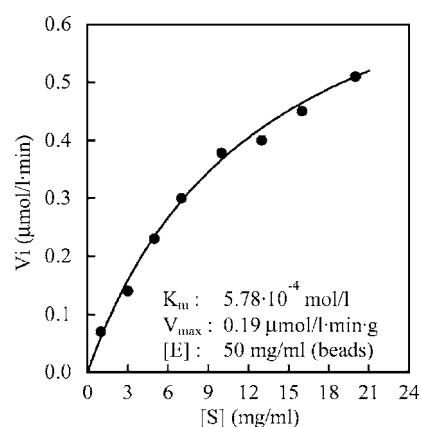


Fig. 9. Kinetics of immobilized XIX protease on chitosan big beads of batch no. 1 in casein hydrolysis, stirred reactor, evolution of V_i ($[S]$) and determination of V_{max} and K_m (●● experimental values; (—) non-linear regression).

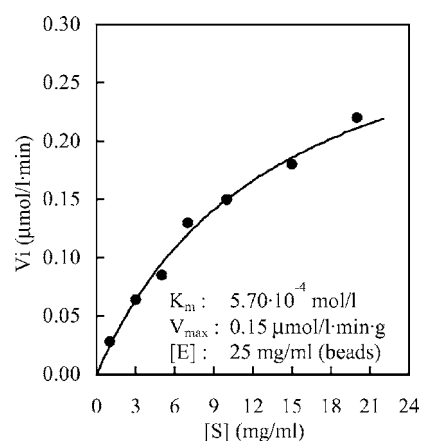


Fig. 10. Kinetics of immobilized XIX protease on chitosan big beads of batch no. 1 in casein hydrolysis, torus reactor, evolution of V_i ($[S]$) and determination of V_{max} and K_m (●● experimental values; (—) non-linear regression).

observed for immobilized enzyme in Figs. 9 and 10, the initial reaction rate is proportional to the enzyme concentration, confirming Michaelian behaviour of hydrolysis. Values of K_m for free and immobilized enzyme are close, indicating that the specificity of enzyme is not affected by the immobilization procedure. Conversely, the maximum apparent reaction rate is lower for the immobilized enzyme. This was due to the decreased activity of the immobilized enzyme

Table 3
Hydrolysis of casein by protease XIX: kinetics parameters

| Enzyme configuration | K_m (mol/l) ^a | V_{max} ($\mu\text{mol/l min g}$) ^b |
|----------------------------|----------------------------|--|
| Free, 10 ml reactor | 4.17×10^{-4} | 3.92 |
| Immobilized, 10 ml reactor | 5.78×10^{-4} | 0.19 |
| Immobilized, torus reactor | 5.70×10^{-4} | 0.15 |

^a Casein average weight 24000 Da.

^b Immobilization rate: 92.8 mg enzyme/1000 mg bead.

and by a limited access for substrate. Accessibility is conditioned by the type of support, the enzyme and by the immobilization procedure. No differences of V_{\max} were observed between the stirred and the torus reactors, confirming the applicability of the latter.

4. Conclusion

Chitosan beads of 1–3 mm diameter were a suitable support for enzyme immobilization in the torus reactor. These showed satisfactory mechanical, chemical and thermal resistances. However, crosslinked beads were more prone to erosion and fragmentation by contact with the propeller blades. Covalent immobilization by glutaraldehyde allowed binding of protease XIX from *A. sojae*. Kinetic parameters for free and immobilized enzyme are in the same order of magnitude denoting the same specificity for the substrate, but activity of the immobilized protease was only 1/20 that of the free enzyme and maximum apparent reaction rate was lower. Nevertheless a high degrees of hydrolysis were obtained for casein in the stirred tank and in the torus reactor and the possible multiple reuse of the larger beads show the potential interest of immobilized enzyme reactors. After these promising results, the next step of study will be to drive an open configuration with the torus reactor containing immobilized enzymes working in continuous flow.

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Appendix A. Nomenclature

| | |
|------------------|---|
| d_1 | blades external diameter |
| d_2 | blades internal diameter |
| DH | degree of hydrolysis |
| D_t | torus reactor internal diameter |
| [E] | enzyme concentration |
| h | number of peptide bonds cleaved during hydrolysis |
| h_0 | total number of peptide bonds |
| k_{cat} | apparent catalytic constant |
| k_i | rate constants |
| K_m | apparent kinetic constant |
| L_t | torus reactor average length |
| [S] | substrate concentration |

| | |
|------------|------------------------|
| V_i | apparent reaction rate |
| V_{\max} | maximum reaction rate |
| ϕ | blades angle |

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