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Immobilization of the Serine Protease from *Thermomonospora fusca* YX

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The heat-stable serine protease from *Thermomonospora fusca* strain YX was immobilized by cyanogen bromide coupling to Sepharose-4B. The immobilized protease hydrolyzed low molecular weight substrates in accordance with Michaelis–Menton kinetics at 25 °C, while at 65 °C diffusion appeared to be rate-limiting. Immobilization of the protease increased its half-life at 85 °C, by a factor of 3 at pH 6.25 and by a factor of 5 at pH 8.50. A packed column of the immobilized protease efficiently hydrolyzed both bovine serum albumin and β-lactoglobulin. Hydrolysis was monitored by an acid precipitation method as well as colorimetric analysis of terminal amino groups.

The immobilization of proteases from different sources has been widely studied in recent years (Clark and Bailey, 1983; Kumakura et al., 1983; Nakanishi et al., 1985; Skachova and Kucera, 1983). Few reports have addressed the successful immobilization of heat-stable proteases, however, despite the potential benefit of this technology to industry (Cowan and Daniel, 1982; Hultin, 1983).

A thermostable alkaline protease was recently purified from *Thermomonospora fusca* strain YX (Gusek and Kinsella, 1987). The novel protease may be applied to the production of protein hydrolysates for food and medical applications (Gusek and Kinsella, 1988). An immobilized form of the protease in a packed-bed reactor would facilitate the production of a uniform protein hydrolysate, preclude protease autolysis, and circumvent the need to recover the protease from the product eluate. Immobilization of the enzyme to a polysaccharide support using cyanogen bromide was selected because this procedure is amenable to proteins possessing few lysine groups (Axen et al., 1967; Stolzenbach and Kaplan, 1976). The *T. fusca* protease contains a single lysine residue (Kristjansson, 1988).

The objective of this research was to examine the effect of CNBr immobilization on the kinetics and thermostability of the protease from *T. fusca* and the evaluate the proteolytic and esterolytic activity of the immobilized enzyme toward various substrates.

REAGENTS

Sepharose-4B, cyanogen bromide activated Sepharose-4B, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPF-pNA), bovine serum albumin (BSA), ovalbumin grade V, equine skeletal myoglobin, hen egg white lysozyme, and casein (sodium salt) were purchased from Sigma Chemical Co., St. Louis, MO. Trinitrobenzenesulfonic acid (TNBS) was purchased from the Aldrich Chemical Co., Milwaukee, WI. Buffer salts, glycerol, formic acid, trichloroacetic acid (TCA), and diethylamine were purchased from Mallinckrodt Inc., Paris, KY. Sephadex G-25 and CM-Sephadex C-50 resins were purchased from Pharmacia Inc., Piscataway, NJ. β-Lactoglobulin (βLG) was purified in this laboratory by the method of Armstrong et al. (1967).

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PROCEDURE

Protease Production and Purification. *T. fusca* YX was cultured and the protease purified as described by Gusek and Kinsella (1987), with the following exceptions. The cultures were grown in 500-mL batches on 1% Solka-Floc SW-40 (James River Corp.) in 2.8-L baffled Fernbach shake flasks (Bellco Glass Inc.). The culture fluids were filtered through a No. 1 cellulose filter (Whatman Ltd.), and the filtrate was adjusted to 50% saturation with $(NH_4)_2SO_4$ at 2 °C. The precipitated proteins were collected by centrifugation and desalted on a Sephadex G-25 column. The salt-free fractions were then diluted with 20 mM Na_2HPO_4 buffer at pH 8.0 and applied to a CM-Sephadex C-50 column equilibrated in the same buffer. Phosphate buffer was used instead of Tris-HCl to preclude interference from Tris amino groups during the subsequent CNBr-immobilization procedure and protein assays. After nonbinding proteins were washed from the CM-Sephadex, the bound proteins were eluted with a linear gradient of 0.5 M NaCl in 20 mM Na_2HPO_4. The major peak containing proteolytic activity (eluting at 100 mM NaCl) was collected for immobilization and SDS-polyacrylamide gel electrophoresis. The remaining fractions were stored in 50% glycerol at −20 °C.

Protease Immobilization. The procedure for CNBr immobilization of the protease followed that of Stolzenbach and Kaplan (1976). One gram of cyanogen bromide activated Sepharose-4B was hydrated in 10 mL of 100 mM Na_2HPO_4 (pH 8.50). To this was added 25 mL of the major protein peak from the CM-Sephadex eluent, containing 0.75 mg/mL protein in 20 mM Na_2HPO_4/100 mM NaCl (from ion exchange). After 40 h, the Sepharose-4B was washed four times with 20 mL of phosphate buffer and one final time with 20 mL of buffer plus 2% diethylamine to occupy unreacted sites, as suggested by Keung et al. (1982). The immobilized enzyme was initially stored in 100 mM Na_2HPO_4 (pH 8.50), but the protease was not stable at this pH, decaying with a half-life of 7 days. The buffer was replaced with 100 mM Na_2HPO_4 (pH 6.50) (2 pH units below the optima for this enzyme), where no further enzyme degradation was observed. All operations were conducted at 6 °C.

Assays. Proteolytic activity was quantified by the casein assay described by Gusek and Kinsella (1987). In addition, a colorimetric assay for esterase activity with SAAPF-pNA as substrate was used as suggested by DelMar et al. (1979). SAAPF-pNA concentrations were determined by molar extinction coefficients of 1400 cm^{-1} at 315 nm (unreacted) and 8480 cm^{-1} at 410 nm (hydrolyzed) (Kristjansson, 1988). The initial rate of hydrolysis of SAAPF-pNA in 50 mM Tris-HCl (pH 8.50) was measured by the change in absorbance at 410 nm with a Cary 219 recording spectrophotometer (Varian Instruments, Sunnyvale, CA).

Protein concentration was quantified by the colorimetric method of Lowry et al. (1951) for proteins adsorbed to surfaces. Protein samples were mixed with equal volumes of 2.0 M NaOH and the resultant mixtures allowed to stand for 60 min. Then, 2.0 mL of copper reagent (2% Na_2CO_3, 0.02% sodium tartrate, 0.01% $CuSO_4$) was added to 0.20 mL of the protein samples. Folin reagent (0.20 mL) was added after exactly 10 min, and the absorbance of the solution at 750 nm was measured after 30 min more, against ovalbumin as the standard.

An assay for concentration of external amine groups was based on the TNBS assay of Wang et al. (1976). Protein samples (1.0 mL) in 100 mM $Na_2B_4O_7$ were mixed with 0.20 mL of 15 mM TNBS. These reacted for exactly 30 min at 37 °C in the dark, with the reaction quenched by the addition of 2.0 mL of 88% formic acid. The concentration of free amino groups was determined by absorbance at 340 nm with BSA as a standard, assuming 60 free amino groups per BSA molecule (Dayhoff, 1978).

The concentration of Sepharose-4B was determined from the turbidity of the gel suspension at 660 nm. Well-mixed suspensions of Sepharose-4B in Na_2HPO_4 buffer generated a standard curve with good linearity ($R^2 = 0.99$) up to at least 20 mg of dry gel/mL.

Saturation Kinetics. Initial reaction rates for both the free and CNBr-immobilized proteases were determined at various concentrations of SAAPF-pNA and at two different temperatures, 25 and 65 °C. At 25 °C, SAAPF-pNA solutions from 0.031 to 1.50 mM in 50 mM Tris-HCl (pH 8.50) were hydrolyzed by either 23 μg/mL native protease or 110 μg/mL CNBr-immobilized protease (25 mg of dry Sepharose-4B/mL). At 65 °C, SAAPF-pNA solutions from 0.047 to 8.84 mM in 50 mM Tris-HCl (pH 8.50) were hydrolyzed by either 23 μg/mL native protease or 85 μg/mL CNBr-immobilized protease (19 mg of dry Sepharose-4B/mL). Saturation parameters were determined by Eadie–Hofstee plots with linear regression (Clark and Bailey, 1983).

Thermal Stability. Thermal stability was assessed from the initial reaction rates for both the native and CNBr-immobilized proteases after prolonged heating at 85 °C. The enzyme was prepared in either of two buffers, 0.20 M Tris-HCl (pH 8.50) (85 °C) or 0.20 M Na_2HPO_4 (pH 6.25) (85 °C), to final concentrations of either 23 μg/mL native protease or 185 μg/mL CNBr-immobilized protease. Separate aliquots were heated in a hot water bath at 85 °C from 5 to 60 min with residual proteolytic activity quantitated at 25 °C using 0.50 mM SAAPF-pNA in 50 mM Tris-HCl (pH 8.50). Half-lives were determined from semilogarithmic plots of thermal deactivation curves, fit by linear regression.

Proteolysis. Approximately 0.70 g (1.81 mL) of Sepharose containing the CNBr-immobilized protease was packed into a small column (0.6 cm × 6.8 cm) at 25 °C. Solutions of BSA (2.08 mg/mL in 50 mM $Na_2B_4O_7$ (pH 8.50)) and βLG (2.16 mg/mL in 100 mM $Na_2B_4O_7$ (pH8.50)) were eluted through the packed gel at flow rates varying from 0.07 to 0.7 mL/min. The extent of proteolysis was determined by two methods. TCA was added to the effluent to a final concentration of 5% and the precipitated protein removed by centrifugation. Samples of βLG were first diluted 3:10 in 100 mM $Na_2B_4O_7$. The extent of proteolysis was expressed as the concentration of acid-soluble fragments as determined by absorbance at 280 nm. In the second assay method, the production of external amine groups was quantified by the TNBS assay described above (Wang et al., 1976).

Table I. Purification of the Protease from *T. fusca* YX[a]

no.	purification step	vol, mL	total act., units	sp act., units/mg	rec, %
1	redissolved 0–50% satn $(NH_4)_2SO_4$ pellet	75	4689		100
2	Sephadex G-25 (fine)	420	4566	2.6	97
3	CM Sephadex C-50 (minor peak)	77	978	41.3	21
	CM Sephadex C-50 (major peak)	75	2320	36.2	49

[a] Total activity is expressed as milligrams of casein hydrolyzed per minute. Specific activity is expressed as milligrams of casein hydrolyzed per minute per milligram of enzyme.

RESULTS

Protease Purification. Purification by cation-exchange chromatography resulted in a 70% recovery of proteolytic activity (by casein assay) from the redissolved ammonium sulfate pellet (Table I). This recovery was an improvement upon the 33% recovery from the original procedure, which employed freeze-drying as a means of enzyme concentration (Gusek and Kinsella, 1987). The protease preparation was shown to be pure by SDS–polyacrylamide gel electrophoresis (Figure 1). The lone band corresponded to a molecular mass of about 19 kDa, consistent with mass spectrometry and amino acid analyses (Kristjansson, 1988). The original molecular mass value of 14.5 kDa (Gusek and Kinsella, 1987) underestimated the actual size of the protease due to incomplete enzyme denaturation.

Protease Immobilization. After immobilization the concentration of immobilized protein on Sepharose-4B was 4.3 mg/g, corresponding to a 22% recovery of total protein. A maximum reaction rate of 0.65 μmol/s per mg of CNBr-immobilized enzyme was obtained with

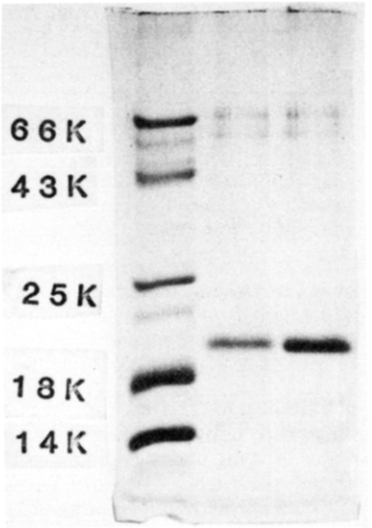

Figure 1. SDS–polyacrylamide gel electrophoresis of the purified protease from *T. fusca* YX. The SDS–polyacrylamide gel was prepared by a 10% running gel and stained with Coomassie Brilliant Blue R. Standards (lane 1) are bovine serum albumin, ovalbumin, chymotrypsinogen A, β-lactoglobulin, and lysozyme (M_r 66, 43, 26, 18, and 14K, respectively). Purified *T. fusca* YX protease is in lanes 2 (minor peak) and 3 (major peak, used in this study). The faint bands in lanes 2 and 3 were carried over from lane 1.

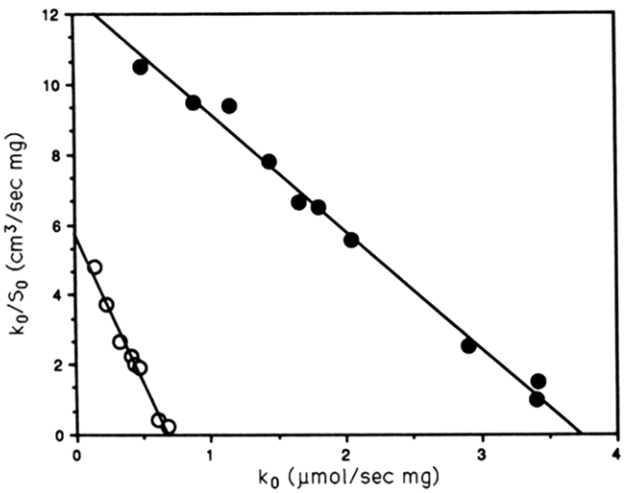

Figure 2. Eadie–Hofstee plot of the protease from *T. fusca* YX, using SAAPF-pNA as the substrate. The reactions were conducted at (○) 25 °C and (●) 65 °C. Saturation parameters were determined as k_{cat} = 0.67 μmol/s per mg, K_m = 0.12 mM (25 °C) and k_{cat} = 3.66 μmol/s per mg, K_m = 0.30 mM (65 °C).

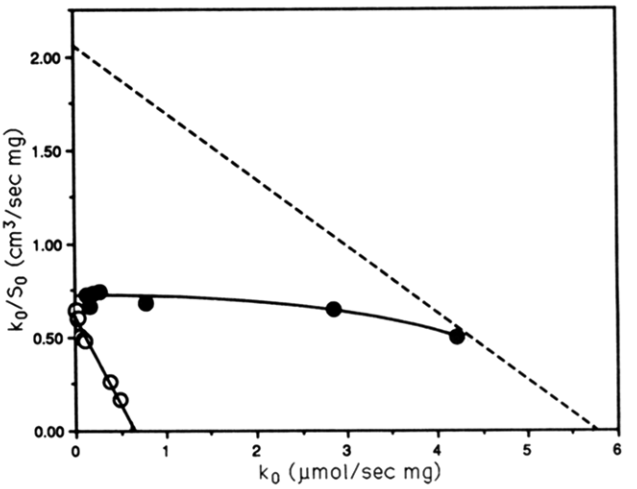

Figure 3. Eadie–Hofstee plot of the CNBr-immobilized protease from *T. fusca* YX, using SAAPF-pNA as the substrate. The reactions were conducted at (○) 25 °C and (●) 65 °C. Saturation parameters at 25 °C were determined as k_{cat} = 0.65 μmol/s per mg and K_m = 1.12 mM. A first-order reaction rate constant at 65 °C of k_f = 0.73 cm³/s per mg was determined. An estimation of intrinsic saturation parameters at 65 °C (- - -) is also plotted, using k_{cat} = 5.77 μmol/s per mg and K_m = 2.8 mM. From the intercepts, a reaction efficiency at 65 °C of η = 0.35 was estimated.

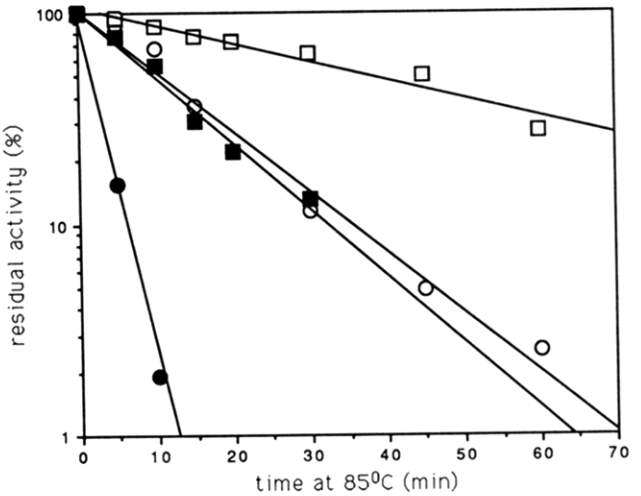

Figure 4. Thermal inactivation of native and CNBr-immobilized protease from *T. fusca* YX. Residual activity after heating at 85 °C was determined using 0.50 mM SAAPF-pNA at 25 °C and pH 8.50. Points shown are free enzyme heated at (○) pH 6.25 and (●) pH 8.50 and CNBr-immobilized enzyme at (□) pH 6.25 and (■) pH 8.50.

SAAPF-pNA at 25 °C, corresponding to 96% of the specific activity of the soluble enzyme (0.67 μmol/s per mg). The free protease had a specific activity of 30 mg of casein hydrolyzed/min per mg of protein while the CNBr-immobilized protease had a specific activity of 9.5 mg of casein hydrolyzed/min per mg of immobilized protein (a 3-fold reduction in the specific activity toward casein).

Saturation Kinetics. The kinetic data for the native protease at both 25 and 65 °C were found to be linear on the Eadie–Hofstee plot (Figure 2). The maximum reaction rate and saturation concentration are highly temperature-dependent. At 25 °C the protease had a maximum specific reaction rate k_{cat} = 0.67 μmol/s per mg of enzyme and K_m = 0.12 mM, while at 65 °C k_{cat} = 3.66 μmol/s per mg of enzyme and K_m = 0.30 mM.

The CNBr-immobilized protease also followed saturation kinetics at 25 °C (Figure 3), showing an apparent maximum specific reaction rate k_{cat} = 0.65 μmol/s per mg of enzyme and apparent K_m = 1.12 mM. At 65 °C the CNBr-immobilized protease deviated from ideal Michaelis–Menton kinetics, although an apparent first-order reaction rate k_f = 0.73 cm³/s per mg was determined, probably the result of diffusion limitation of SAAPF-pNA.

A mathematical treatment of this diffusion limitation is consistent with the analysis of Clark and Bailey (1983). First, since the reaction is limited to the region within the porous Sepharose-4B beads, the bulk reaction rate is inappropriate and should be replaced with the reaction rate per unit bead volume. Assuming that Sepharose-4B has a density of 0.33 g of dry gel/cm³ of wet gel (Sigma), at 25 °C the maximum initial reaction rate per unit gel volume is calculated as v_{max} = 0.95 μmol/cm³ gel s. Assuming that Sepharose-4B beads have a radius of 80 μm and that SAAPF-pNA has an effective diffusivity of D_{eff} = 3

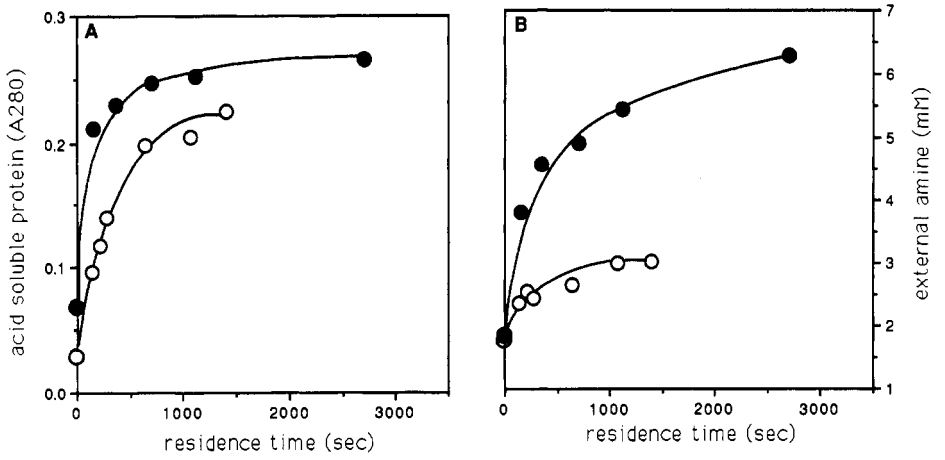

Figure 5. Hydrolysis of bovine serum albumin and β-lactoglobulin by a plug-flow column containing CNBr-protease from *T. fusca* YX. The concentration of protein fragments of BSA (○) and βLG (●) soluble in 5% TCA (A) is expressed as absorbance at 280 nm. The concentration of external amine groups (B) was determined by the TNBS assay.

$\times 10^{-6}$ cm^2/s through the porous gel (Clark and Bailey, 1983), the Thiele modulus is calculated to be $\phi = 1.4$. The Thiele modulus represents the ratio of reaction rate to diffusion rate within a porous catalyst particle, in this case indicating that diffusion is not limiting.

Assuming that the apparent K_m of the immobilized enzyme at 65 °C is 2.5 times that at 25 °C (as it was with free enzyme), a K_m of 2.8 mM is estimated. Projecting this K_m through the maximum observed reaction rate (Figure 3) yields an estimated maximum specific reaction rate of $k_{cat} = 5.8$ μmol/s per mg, or $v_{max} = 8.2$ μmol/cm^3 per s. From these assumptions, a Thiele modulus $\phi = 2.7$ is calculated, within the range of diffusion limitation. The effectness factor (η) is defined as the ratio of the diffusion-limited reaction rate to the rate that would be observed without diffusion limitation. For the diffusion-limited reaction the ratio of k_o/S_o is a constant value (k_f), equal to $\eta k_{cat}/K_m$. A Thiele modulus of 2.7 predicts an effectiveness factor of 0.37. From the estimated k_{cat} and K_m (Figure 3), the apparent first-order rate constant $k_f = 0.73$ cm^3/s per μg gives an estimated effectiveness factor of 0.35, consistent with that predicted by the Thiele modulus. Although this analysis is only approximate, it supports the hypothesis of diffusion limitation at low reaction velocities.

Thermal Stability. CNBr-immobilization enhanced the thermal stability of the protease. At pH 8.50 and 85 °C, the soluble enzyme demonstrates high activity but is susceptible to thermal denaturation and autolysis (Kristjansson, 1988). At pH 6.25, the enzyme has low activity but remains susceptible to thermal denaturation. It was predicted that immobilization would protect the enzyme from autolysis at higher temperatures and pH 8.50. Immobilization increased the half-life of the enzyme at both pH values, from 10.6 to 34.7 min at pH 6.25 and from 1.9 to 9.7 min at pH 8.50 (Figure 4). Prolonged enzyme lifetime at pH 6.25 appears to be due to increased conformational stability after immobilization. It is unclear whether immobilization enhanced enzyme thermostability or minimized autolysis at pH 8.50, both factors of which would increase enzyme lifetime.

Proteolysis. The CNBr-immobilized protease retained activity against both BSA and βLG, with the extent of hydrolysis increasing with prolonged column residence time. While the concentration of acid-soluble peptides approached a maximum value after a column residence time of 500 s (Figure 5A), the concentration of free amino groups continued to increase beyond 1000 s (Figure 5B), because of more extensive hydrolysis of the acid-soluble protein fragments. The maximum extent of hydrolysis represented an additional 40 terminal amines per BSA molecule and an additional 53 per βLG molecule. Three other proteins (myoglobin, lysozyme, ovalbumin) were subjected to proteolysis under similar conditions, but significantly lower rates of proteolysis were observed (data not shown).

DISCUSSION

In order to maximize the amount of enzyme coupled to the activated support, an excess of protease was used during the immobilization procedure. The efficiency of enzyme binding increased by 30% from the 22% observed when the enzyme concentration was reduced by 1 order of magnitude (unpublished data). Quantitative recovery of enzyme specific activity toward SAAPF-pNA (96%) after immobilization supports the notion of substrate diffusional limitation. When the soluble and bound enzymes were evaluated against bulky high molecular weight substrates such as casein, the immobilized protease had a reduced specific activity of 3-fold. External diffusional limitations or internal diffusional restrictions accounted for the 45- to 300-fold reduced activities of three other immobilized proteases toward whole casein (Beeby, 1979). The disparity in specific activity between soluble and immobilized proteases becomes less apparent when low molecular substrates are tested (Cheetham, 1985).

The unbound protease obeyed Michaelis–Menton kinetics for the single-site SAAPF-pNA. At higher temperatures (65 °C), the deviation from ideal Michaelis–Menton kinetics of the immobilized enzyme, as shown by the Eadie–Hofstee plot, may also be attributed to substrate diffusional limitation. The reaction efficiency of 0.35 (estimated from the plot) is in close agreement with the effectiveness factor of 0.37 (estimated with the Thiele modulus).

The successful immobilization of the protease from *T. fusca* YX is the first step in the development of a continuous-flow column reactor. Diffusional limitations at higher temperatures, even for small substrates like SAAPF-pNA, pose a potential problem for a protein hydrolysis column reactor comprised of immobilized protease beads. However, the packed column was found to be effective in hydrolyzing both BSA and βLG at 25 °C. Future research will examine the feasibility of coupling the protease to alternative supports to permit higher operational temperatures and flow rates.

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SYMBOLS USED

k_0	initial specific reaction rate (μmol/s per mg of enzyme)
k_{cat}	maximum initial specific reaction rate (μmol/s per mg of enzyme)
k_f	apparent first-order specific reaction rate (cm^3/s per mg of enzyme)
S_0	initial substrate concentration (mM)
K_m	substrate concentration (mM) at $V_{max}/2$
ν_{max}	initial reaction rate per unit bead volume (μmol/s per cm^3 of gel)
ϕ	Thiele modulus
η	reaction effectiveness factor

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