SHORT COMMUNICATION

Kinetic characterization of sequencing grade modified trypsin

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Prior to analysis by mass spectrometry, protein samples are often digested. Maximizing the peptide yield from digestion can increase the number of peptides detected and the confidence in protein identification. To determine the optimal conditions for digestion, the Michaelis-Menten kinetic parameters for Promega sequencing grade modified trypsin were measured over a range of temperatures and pHs. The results indicate that an increase in digestion temperature above 37°C, the temperature traditionally used in digestion methods, could offer an increase in peptides detected.

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MS is used commonly for protein identification and characterization. Prior to MS analysis, a protein sample is often digested chemically or enzymatically. Optimizing the digestion procedure to maximize the peptide yield has been a research area of active interest [1–5]. Here, peptide yield refers to the total number of peptides produced and their intensities. Maximizing the peptide yield can increase the number of peptide peaks in the MS and MS/MS spectra, and subsequently increase the confidence in the protein identification. The most commonly used enzyme for pre-MS digestions is modified trypsin. Modified trypsin is altered to reduce autolysis and to inhibit the chymotryptic activity of any trypsin autolysis peptides [6]. Several studies have indicated that these chemical modifications can change the kinetic properties of trypsin [1, 7]. A study by Havlis *et al.* [1] measured the quantity of peptides produced by in-gel digestion of BSA with modified trypsin, using a range of digestion temperatures. They found that the maximum peptide yield occurred at a higher temperature for modified trypsin than for unmodified trypsin. The kinetic parameters for modified trypsin, however, have not been published previously. In this study, the Michaelis-Menten kinetic parameters for modified trypsin were measured over a range of temperatures and pHs. The kinetic parameters were used in the Michaelis-Menten model to compare the amount of protein that would be digested under varying digest conditions. The rate of inactivation of modified trypsin (due to autolysis and denaturation) was also measured at several temperatures.

The modified trypsin used in these experiments was purchased from Promega (sequencing grade porcine, lot# 16949301, specific activity 17 000 $U \cdot mg^{-1}$). This trypsin preparation is modified by the supplier using reductive methylation and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) treatment. To create the labeled substrate, a casein solution (Sigma, (St. Louis, MO, USA) bovine, 10 $mg \cdot mL^{-1}$ in 0.1 M sodium bicarbonate) was mixed with 1/10th volume of FITC solution (Molecular Probes, Eugene, OR, USA; F-143, 10 $mg \cdot mL^{-1}$ in DMSO) and incubated at room temperature for 1 h. Unbound FITC was separated from FITC-casein using a PD-10 column containing Sephadex (Amersham Biosciences, Uppsala, Sweden) following manufacturer's instructions. The FITC-casein was eluted in a solution of 100 mM sodium phosphate (70:30 ratio of monobasic to dibasic).

The trypsin activity assays are based on the protocol published by Twining [8]. The FITC-casein was diluted with 0.2 M Tris-HCl (pH = 7.8) to the desired concentration. Trypsin (1.46 $\mu g \cdot mL^{-1}$) in 0.2 M Tris-HCl (pH = 7.8) was mixed with the diluted FITC-casein in a 1:1 ratio. The digesting trypsin/

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Abbreviations: L-BAPNA, benzoyl L-arginine *p*-nitroanilide hydrochloride; **DACM**, *N*-(7-dimethylamino-4-methylcoumarinyl)-maleimide; **TPCK**, *N*-tosyl-L-phenylalanine chloromethyl ketone

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casein solution was incubated at 37°C while shaking for 3 min. At this point, an aliquot of the digest solution was removed and mixed with 5% TCA to precipitate undigested FITC-casein (FITC-casein and TCA solutions in a ratio of 1:2.5). The precipitation occurred for 1 h at room temperature and then overnight at 4°C. The solution was then centrifuged, and the supernatant containing the digested FITC-casein was removed. Three 80 μL aliquots of the supernatant were added to separate wells of a glass-bottomed 96-well plate. To raise the pH, 120 μL of 0.5 M Tris-HCl (pH = 8.8) were added to each well. The fluorescence in each well was then measured using a fluorescent laser scanner (excitation wavelength: 473 nm, emission filter: 520 nm, FLA-3000, Fujifilm). The integrated fluorescence intensity of each well was used with a standard curve to determine the concentration of digested FITC-casein. A total of six measurements were made for each of the three FITC-casein concentrations tested: 6.0, 12, and 20 μM. To investigate the effects of temperature and pH on digestion, the incubation temperature and the pH of the 0.2 M Tris-HCl solution were modified.

The data for the three FITC-casein concentrations were used in a Hanes-Woolf plot to calculate the Michaelis-Menten kinetic parameters K_m and k_{cat}. These parameters were used with the differential equations representing Michaelis-Menten digestion [9] to predict the concentration of digested casein as a function of time. The predicted percentage of casein digested after 1 h of digestion (using an initial casein concentration of 1.2×10^{-4} M and a trypsin concentration of 6.0×10^{-8} M) was used in subsequent comparisons.

The rate of inactivation of modified trypsin digesting at several temperatures was also investigated. For each temperature tested, the trypsin activity was measured at several time points using benzoyl L-arginine *p*-nitroanilide hydrochloride as a substrate (L-BAPNA, Sigma) [10]. L-BAPNA is a chromogenic substrate that releases *p*-nitroaniline when digested with trypsin. The L-BAPNA was dissolved in DMSO (43.5 mg·mL^{-1}) and then diluted 1:100 in 0.05 M Tris-HCl/0.02 M $CaCl_2$ (pH = 8.2). Trypsin (1.46 μg·mL^{-1} in 0.2 M Tris-HCl, pH = 7.8) was heated to the desired temperature while shaking. At each time point, an aliquot of the heated trypsin solution was added to L-BAPNA solution and water in a ratio of 1:1:10 (trypsin:water:L-BAPNA). The reaction between the trypsin and L-BAPNA was allowed to take place at room temperature for 2 h. The digestion was stopped by the addition of 30% acetic acid and the A at 410 nm was then measured using a spectrophotometer (UV1201, Shimadzu). The percent of remaining activity at *t* minutes was found by dividing the absorbance of the sample taken at time *t* by the A of the sample taken at 0 min. The experiment was performed three times for each data point and the average and standard deviations were calculated.

In the experiments to determine the kinetic constants of trypsin, FITC labeled casein was used instead of a synthetic substrate because the goal was to determine the optimal conditions for the tryptic digestion of proteins. The kinetic constants calculated for each of the tested conditions are shown in Table 1. When interpreting these kinetic parameters, it should be noted that casein does have more than one tryptic cleavage site. However, the concentration of casein was at least 1000 times that of trypsin and the initial rate of digestion was used to calculate the parameters. Therefore, it is assumed that the digestion products represent casein molecules that have only been cleaved once. The high ratio of FITC-casein to trypsin also helps to ensure that the Michaelis-Menten assumption of no enzyme consumption, in this case due to autolysis, is valid. A previous study using unmodified bovine trypsin to digest casein labeled with FITC and *N*-(7-dimethylamino-4-methylcoumarinyl)-maleimide (DACM) at 35°C and pH = 7.5 reported a K_m of 1.6 ± 0.2 μM and a k_{cat} of 0.72 s^{-1} [11]. These reported values are on the same order of magnitude as those in Table 1.

Table 1. Measured Michaelis-Menten kinetic constants for the digestion of FITC-casein by modified porcine trypsin

pH	*T* (°C)	K_m (μM)	k_{cat} (s^{-1})
7.8	58	2.98 ± 0.29	0.48 ± 0.01
7.8	48	3.54 ± 0.17	0.48 ± 0.01
7.8	37	3.33 ± 0.15	0.44 ± 0.01
7.8	30	0.86 ± 0.17	0.35 ± 0.01
7.0	37	0.15 ± 0.19	0.32 ± 0.01
7.5	37	2.09 ± 0.14	0.40 ± 0.01
7.8	37	3.33 ± 0.15	0.44 ± 0.01
8.1	37	2.15 ± 0.43	0.44 ± 0.01
8.6	37	1.60 ± 0.13	0.43 ± 0.01

Figure 1 shows the percentage of casein that is predicted to be digested after 1 h under each of the temperature and pH conditions in Table 1. The temperature resulting in maximum digestion is predicted to occur between 50 and 55°C. This agrees with the previous data suggesting that the maximum activity of modified trypsin occurs between 50 and 65°C [1]. These temperatures are higher than the optimal temperature of unmodified trypsin, which is between 35 and 45°C [1, 12]. Pre-MS tryptic digestions are often performed at *ca.* 37°C [2–5, 13], which is closer to the optimal temperature of unmodified trypsin.

The optimal pH for the modified trypsin, from Fig. 1B, is slightly higher than 8.1. The study using unmodified trypsin digesting casein labeled with FITC and DACM found the optimal pH to be near 7.0 [11]. This suggests that the modifications may increase the stability of the trypsin at higher pHs. The manufacturer's information for the modified trypsin states an optimal pH range of 7.0–9.0 and current digest protocols commonly use a digestion pH between 7.8 and 8.1 [2–5, 13].

At the substrate concentrations normally encountered in an in-gel digest, trypsin autolysis does occur and the trypsin peptides can suppress the sample peptides in the mass spectrum. The change in trypsin activity over time was

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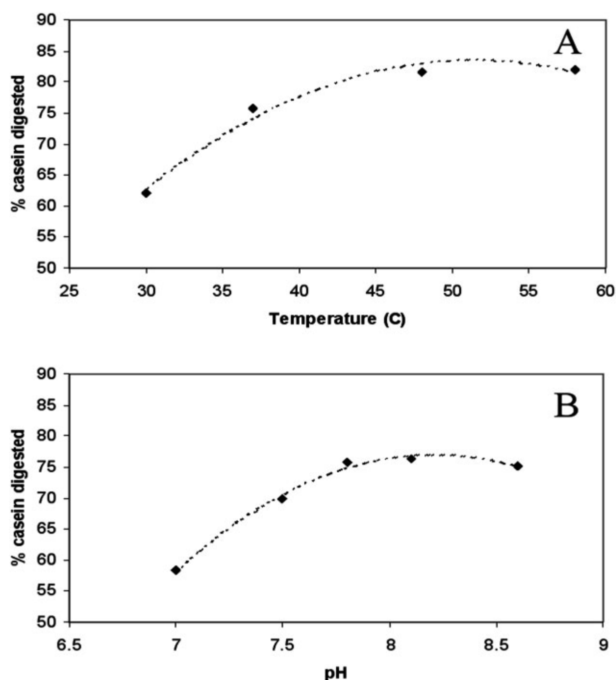

Figure 1. Percentage of casein digested after 1 h using kinetic constants from Table 1 in a computer model. (A) Change in percentage of digested casein over a range of temperatures at pH = 7.8. (B) Change in percentage of digested casein over a range of pH at 37°C.

measured at several temperatures. The average values from the three experiments are shown in Fig. 2. At all temperatures tested, there is a decrease in activity over time. This is presumably due to inactivation by autolysis and possibly thermal denaturation at the higher temperatures. The decrease in activity is more pronounced with increasing temperature. At 58°C the enzyme rapidly declines to 40% activity after only 2 h. This is in agreement with the results reported by Havlis *et al.* in which heavy contamination from trypsin autolysis peptides was observed in the mass spectra of digests performed at 58°C for more than 1 h [1].

The kinetic modeling predictions for casein digestion at multiple pH (Fig. 1B) suggest that the commonly used digestion pHs (7.8–8.1) are in the optimal range for modified trypsin. The results from modeling the casein digestion over a range of temperatures (Fig. 1A) suggest that the commonly used digestion temperature (37°C) could be raised to increase the extent of protein digestion. Although the maximum amount of digestion was predicted by the kinetic modeling to occur between 50 and 55°C, the measurement of the percentage of remaining trypsin activity over time indicates that there could be significant autolysis near these temperatures. Digestions at these higher temperatures may therefore require a method, such as LC-MS, that separates the peptides prior to MS analysis to prevent signal suppression by trypsin autolysis peptides. The results of this study indicate that a smaller increase in temperature, possibly to about 48°C, could provide an increase in protein digestion

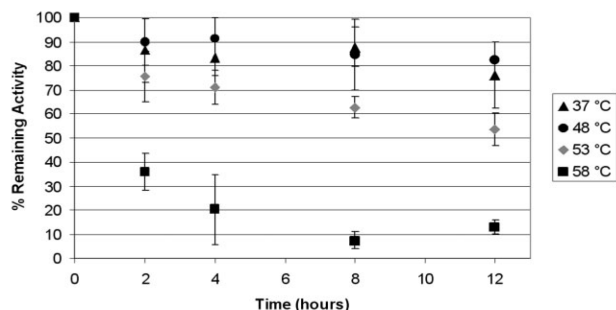

Figure 2. Percent of remaining trypsin activity over time. Each data point represents the average of three trials and the error bars show the standard deviation.

without a significant increase in autolysis. This is the first study to have measured the kinetic parameters for the modified trypsin commonly used in pre-MS digestions. Although this study has focused on the effects of temperature and pH, the rate constants from this study can also be used to determine the effects of other changes such as digestion time and sample to substrate ratio.

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