New Colorimetric Method for the Detection and Quantitation of Proteolytic Enzyme Activity†

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A new and sensitive colorimetric assay was developed to detect and quantitatively measure proteolytic enzyme activity using 4-(dimethylamino)azobenzene-4′-sulfonyl (DABS)–casein as the substrate. The enzyme assays were linear over enzyme:DABS–casein ratios (E:S) 2×10^{-2}–2×10^{-4} and up to 120 min of incubation time. This DABS–casein assay can detect trypsin and chymotrypsin enzyme activities as low as 64.16 and 89.86 ng, respectively (for 120 min of incubation). This sensitivity is comparable to the assays that use fluorescence and radioactive labels. Labeling the casein with DABS-Cl did not change the pH optimum of either trypsin or chymotrypsin. DABS-Cl labeled casein has excellent stability (5 months in solution form at 4 °C and indefinite at −20 °C in dry powder form). The enzyme activities can be measured in the presence of many chemicals commonly encountered in foods and biological buffers by using DABS–casein as the substrate.

Keywords: *Enzyme; colorimetric assay; DABS–casein*

INTRODUCTION

Numerous methods are available for measuring proteolytic activity (Sarath et al., 1989). Such assays include the use of natural protein substrates as well as synthetic peptides depending on the enzyme being assayed and the availability of the substrate. Methods using protein as the substrate typically require the separation of proteolytic products from the assay mixture prior to measurement of either the remaining substrate or the proteolytic products. One of the most commonly used methods for such assays is the measurement of ultraviolet (UV) absorbance of trichloroacetic acid (TCA) soluble peptides generated by the action of the proteinase on a protein substrate (such as milk casein). Typically, the absorbance is measured at 280 nm in this method. This absorbance is due to the absorbance by aromatic amino acids, and consequently the usefulness of the method depends on the ability of the proteinase to liberate the peptides that contain aromatic amino acids from the substrate. To overcome such limitations, chromophoric labeling (Chang et al., 1981; Lin and Chang, 1975; Ladd and Snow, 1993; Kulseth and Helgeland, 1993; Tomarelli et al., 1949) and fluorogenic labeling (Anjuère et al., 1991; Bolger and Checovich, 1994; Farmer and Yuan, 1991; Spencer et al., 1975; Twining, 1984; Sogawa and Takahashi, 1978; Wiesner and Troll, 1982) of the substrate protein are commonly used. The labeled substrate proteins have also been adapted to microassay procedures (Plantner, 1991; Hatakeyama et al., 1992) so that a large number of samples can be assayed rapidly. These microassay procedures typically use microtiter plates and therefore require a microplate reader for measuring the enzyme activities. Additional methods commonly used for proteinase assays include the use of radioisotope labeling of the substrate (Sevier, 1976; Robertson et al., 1988; Rucklidge and Milne, 1990), flow injection analysis (Nicolas et al., 1991), unprocessed X-ray films (Cheung et al., 1991), and electrophoretic methods (Sarath et al., 1989; de Barros and Larkins, 1990).

Many of the above-mentioned methods require expensive reagents and specialized equipment (such as for the fluorometric, radioisotope, and electrophoretic methods), expensive disposal of waste products (such as in radioactive isotope labeling methods), and/or *a priori* knowledge of proteinase specificity (as in cases when synthetic substrates are used). Methods that use radiolabeling or the fluorescent labeling of the substrate are some of the most sensitive methods (typically nanogram to picogram sensitivity of detection) available for measurement of proteolytic activities. There is, however, a need for assay methods that are not expensive and yet highly sensitive for detection and quantitation of proteolytic activities using simple visible spectroscopy. Among the several available chromophoric reagents that can bind with amino acids, peptides, and proteins, DABS-Cl is known to be stable and affords detection in the visible range. Dabsyl derivatives of amino acids at concentration of 10^{-11}–10^{-10} mol have been shown to be stable (Lin and Chang, 1975). Detection and reliable quantitation of as little as 2–5 pmol of dimethylaminoazobenzenesulfonyl–amino acid in the visible range (436 nm) has been reported (Chang et al., 1981). We were therefore interested to learn whether DABS-Cl could be used to derivatize casein and then use DABS–casein as the substrate to detect and quantitate proteolytic enzyme activity in a sensitive manner. This paper reports the findings of such studies and indicates that such an approach is feasible to reliably quantitate proteolytic activity at a picomole level.

MATERIALS AND METHODS

Hammersten casein and dithiothreitol (DTT) were obtained from United States Biochemical, Cleveland, OH. 4-(Dimethylamino)azobenzene-4′-sulfonyl chloride (DABS-Cl) was from Pierce Chemical, Rockford, IL. TLCK-treated chymotrypsin (bovine pancreas, type VII, batch 75-8025), TPCK-treated trypsin (bovine pancreas, type XII, batch 66F-8135), β-mer-

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captoethanol (β-ME), and phytic acid were from Sigma Chemical Co., St. Louis, MO. Galactose, catechol, and tannin were from Nutritional Biochemicals Corp., Cleveland, OH. Calcium chloride ($CaCl_2$) was from J. T. Baker Chemical Co., Phillipsburg, NJ. Phloroglucinol was from Matheson Coleman and Bell Division, East Rutherford, NJ. Sodium chloride (NaCl), sodium ethylenediaminetetraacetic acid (Na-EDTA), sodium dodecyl sulfate (SDS), trichloracetic acid (TCA), acetone, and other chemicals were from Fisher Scientific Co., Orlando, FL, and were of reagent, or better, grade.

Preparation of Dabsylated Casein. Hammersten casein was labeled with DABS-Cl. The average molecular weight (MW) of the casein was calculated to be 23 183 on the basis of the following assumptions: (1) The MWs of α_{S1}, α_{S2}, β, κ, γ_1, γ_2, and γ_3 caseins are, respectively, 23 162, 25 228, 23 980, 19 005, 20 520, 11 822, and 11 557. (2) The average MW of α caseins $[(\alpha_{S1} + \alpha_{S2})/2]$ is 24 420, and that of γ caseins $[(\gamma_1 + \gamma_2 + \gamma_3)/3]$ is 14 633. (3) The total casein in milk accounts for 80% of total milk proteins. (4) On an average α, β, κ, and γ caseins, respectively, account for 52.5%, 31.25%, 11.25%, and 5% of the total casein. Briefly, 10 g of casein was dissolved in 250 mL of distilled deionized (DIDI) water (pH adjusted to 8.2 with 0.1 M NaOH) at 80 °C, and the solution was cooled to 70 °C in a constant-temperature water bath. DABS-Cl (0.412 g in 10 mL of acetone) solution (preincubated at 70 °C) was added to the casein solution (1:3 molar ratio of casein:DABS-Cl). The mixture was incubated for 20 min in a 70 °C constant-temperature water bath. The mixture was stirred every 5 min with a spatula. The ratio of casein:DABS-Cl, the pH, and the time of incubation for this reaction were based on preliminary trials in our laboratory as well as amino acid derivatization data reported by Lin and Chang (1975). At the end of the incubation, 25 g of TCA was added (final TCA concentration of 10% w/v) to precipitate DABS–casein. The mixture was cooled to room temperature (25 °C) and centrifuged (12000g, 4 °C), and the pellet was dispersed in 25 mL of DIDI water and neutralized with 1.0 M NaOH. The resulting solution was dialyzed against DIDI water for 24 h to remove salts and unreacted reagent and then lyophilized. The dry DABS–casein was washed with several aliquots of acetone (total 2 L) to remove residual DABS-hydroxide and DABS-Cl, and the residual acetone was evaporated in a hood at room temperature (25 °C). The dry DABS–casein was homogenized in a blender to obtain a uniform powder and stored in an amber glass bottle in the freezer (−20 °C) until further use.

Wavelength Maximum Determination. Visible and ultraviolet absorbance was recorded using Perkin-Elmer Lambda 3 UV–vis spectrophotometer. An absorbance reading of at least 0.050 above that of the corresponding blank was considered to be the detection limit (D. Brown, Perkin-Elmer, Atlanta, GA, personal communication, 1994).

Unhydrolyzed DABS–Casein. The absorbance spectra were determined for several dilutions of unhydrolyzed DABS–casein in 0.1 M $NaHCO_3$ (0.01–5.0 mg/mL) and were measured between the wavelengths of 400 and 600 nm.

Hydrolyzed DABS–Casein. Absorbance spectra of TCA soluble substances generated by the proteolysis of DABS–casein were determined. The E:S molar ratios were in the range 2×10^{-2}–1×10^{-3}, and the incubation time ranged from 5 to 120 min.

Stability of DABS–Casein. DABS–casein solution (0.5 mg/mL in 0.1 M Tris-HCl, pH 8.1, containing 1 mM NaN_3) and the solid DABS–casein were stored in amber glass bottles at 4 and −20 °C, respectively, and were evaluated for stability by measuring the absorbance spectra and the absorbance at 505 nm, periodically. The solid DABS–casein was dissolved in 0.1 M Tris-HCl, pH 8.1, containing 1 mM NaN_3 at 0.5 mg/mL on the day of evaluation for absorbance measurement. Samples were also visually inspected for microbial growth.

Preparation of Stock Solutions. Assay reagents consisted of 5 mg/mL DABS–casein, 500 μg/mL enzyme, 1 M Tris-HCl, pH 8.1, 400 mM $CaCl_2$, and 60% TCA. Other solutions included 100 mM β-ME, DTT, EDTA, catechol, resorcinol, phloroglucinol, tannic acid, and phytic acid, 1 M sugar solutions (sucrose, fructose, glucose, and galactose), 1 M SDS, and 4 M NaCl.

Proteolytic Enzyme Assay. Typically, assays were done in plastic microcentrifuge tubes in 0.1 M Tris-HCl, pH 8.1, buffer containing 10 mM $CaCl_2$ unless the experiment required the use of other buffer. The final volume was 1 mL for enzyme assays. Substrates and enzymes were in proportion as required. Incubation was at 37 °C in a water bath for the desired time. Typically 0.5 mL of casein substrate (5 mg/mL, both underivatized and DABS–casein) was used (final casein concentration of 2.5 mg/mL assay volume). For different E:S ratios the casein concentration was held constant (2.5 mg/mL final concentration) while the enzyme concentration was varied to obtain desired E:S ratios. Enzyme activity was terminated by adding 0.200 mL of 60% cold (4 °C) TCA (final TCA concentration of 10% w/v). Tubes were then centrifuged at 13600g for 5 min at 25 °C. Absorbance of the supernatant was measured at 510 nm when DABS–casein was the substrate and at 280 nm when unlabeled casein was used as the substrate. All assays, including appropriate blanks, were done in triplicate.

pH Optima of Enzyme Activity. The assay procedure was the same as described under Proteolytic Enzyme Assay. E:S molar ratio of 6.66×10^{-3}, buffer pH range of 6.53–9.00 (sodium phosphate buffer at pH 6.53, 6.97, 7.51, and Tris-HCl buffer at pH 8.12, 8.53, and 9), and incubation time of 30 min were used in these assays.

Assay Linearity and Sensitivity. The enzyme assay procedure was the same as described under Proteolytic Enzyme Assay. E:S ratios up to 2×10^{-4} and incubation time up to 120 min were used. For comparative purposes, assays were done using unlabeled casein, E:S ratio up to 1×10^{-3} (trypsin) and 2×10^{-3} (chymotrypsin), and incubation time of 30 min at 37 °C. Absorbance was measured at 280 nm when unlabeled casein was the substrate.

Influence of Inhibiting Agents on Enzyme Activity. These assays used the same general protocol as under Proteolytic Enzyme Assay. Incubation time was 30 min and E:S ratio was 1×10^{-2}. Interfering substances incorporated in the assay were up to 40 mM β-ME, DTT, and EDTA; 200 mM sucrose, glucose, fructose, or galactose; 20 mM catechol, resorcinol, phloroglucinol, tannin, SDS, or phytic acid; and up to 2.0 M NaCl. These are some of the chemicals commonly present in biological buffers and foods.

Statistics. Data are reported as mean ± standard error of mean (SEM). When appropriate, data were analyzed for significant differences ($p = 0.05$) using Fisher's lsd (protected test) as described by Ott (1977). Correlation coefficients were determined for appropriate data.

RESULTS AND DISCUSSION

Maximum Wavelength. Since spectral properties of DABS–casein have not been reported in the literature, DABS–casein spectra were determined (Figure 1). Generally, as the protein concentration increased, maximum absorbance shifted to higher wavelength region (λ_{max} was 437 nm for 0.01 mg/mL and 510 nm for 5.0 mg/mL). The wide range of λ_{max} observed here is consistent with the data on λ_{max} values reported for DABS–amino acid complexes that ranged from 427 to 476 nm (Lin and Chang, 1975).

Because proteolysis causes release of a heterogeneous mixture of peptides and amino acids (depending on the substrate and the enzyme specificity), we investigated the absorbance characteristics of TCA soluble substances released from DABS–casein hydrolysis by both trypsin and chymotrypsin using a wide range of E:S ratios as well as different incubation times (Figure 2). Regardless of the E:S ratio and the incubation time λ_{max} in all instances was 510 nm. Consequently, all proteolytic assays using DABS–casein as the substrate were monitored at 510 nm.

Storage Stability of DABS–Casein. Both the dry and solution form of DABS–casein had maximum

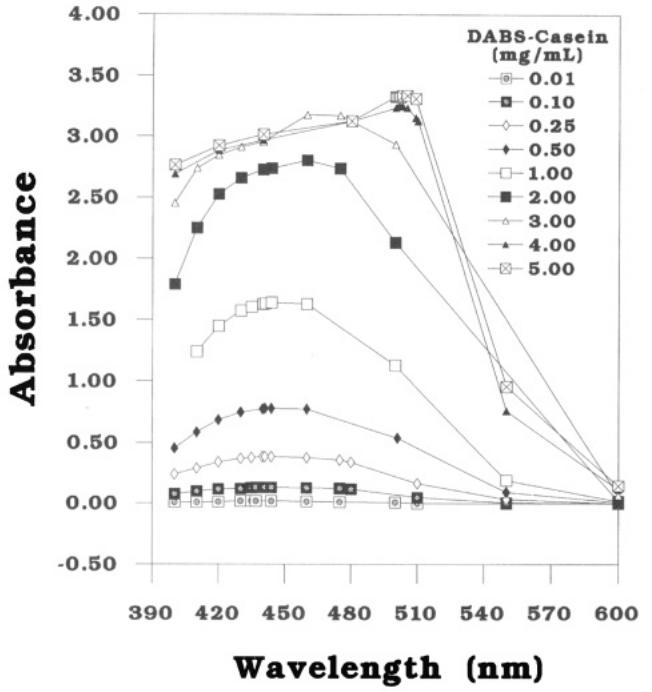

Figure 1. Absorption spectra of DABS–casein (0.01–5.0 mg/mL in 0.1 M $NaHCO_3$).

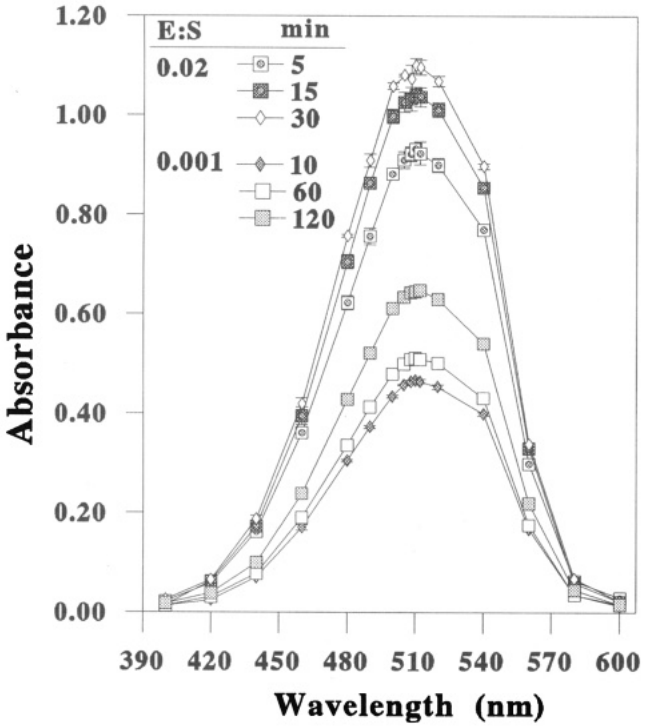

Figure 2. Absorption spectra of TCA soluble products released by the action of trypsin on DABS–casein.

absorbance in the range 437–443 nm and remained in this range for solution up to 5 months and for dry DABS–casein indefinitely. There was no change in absorbance at 505 nm for solutions up to 5 months (absorbance values remained 0.5–0.55 when measured against 0.1 M Tris-HCl, pH 8.1, containing 1 mM NaN_3 as the solvent blank) and for solid form (in the same solvent) indefinitely (absorbance ranged from 0.43 to 0.65). There was visible microbial growth in solutions after 5 months. These data suggested that DABS–casein solutions can be stored for up to 5 months at 4 °C, and the dry form at −20 °C indefinitely. We typically used DABS–casein stock solutions within 2 weeks from preparation day.

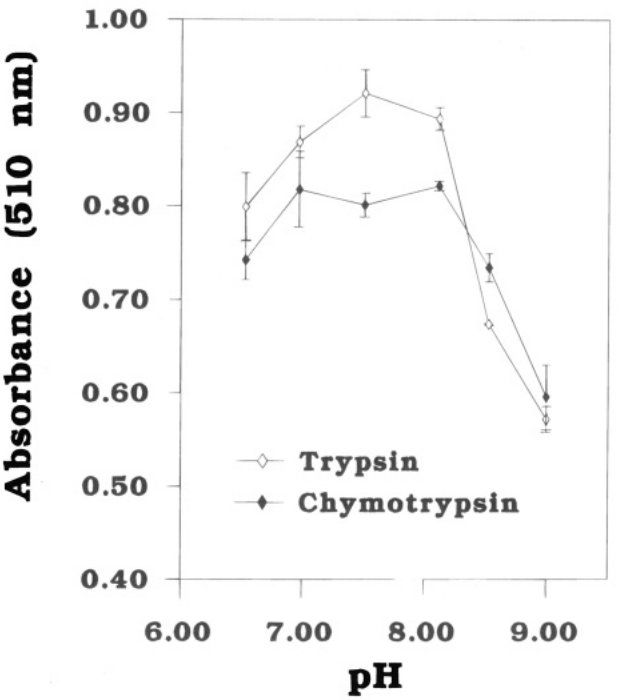

Figure 3. Influence of DABS–casein on pH optima for the trypsin and chymotrypsin assays. E:S ratio was 6.66×10^{-3} for each enzyme, incubation period was 30 min, and absorbance was read at 510 nm.

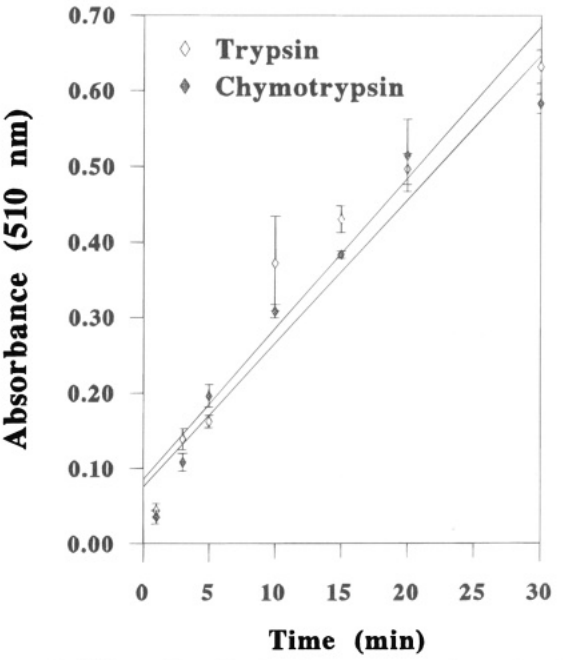

Figure 4. Effect of incubation time on assay linearity. E:S ratio of 2×10^{-3} and incubation time of up to 30 min were used.

pH Optima of Enzyme Activity. The optimum enzyme activity was observed between pH 7.5 and 8.1 for both trypsin and chymotrypsin (Figure 3). This indicated that labeling casein with DABS-Cl did not change the pH optimum of either trypsin or chymotrypsin during the assay.

Assay Linearity. The assay linearity was evaluated using several E:S ratios as well as different incubation times to establish suitable conditions for routine assays. The incubation time ranged from 5 to 30 min for E:S

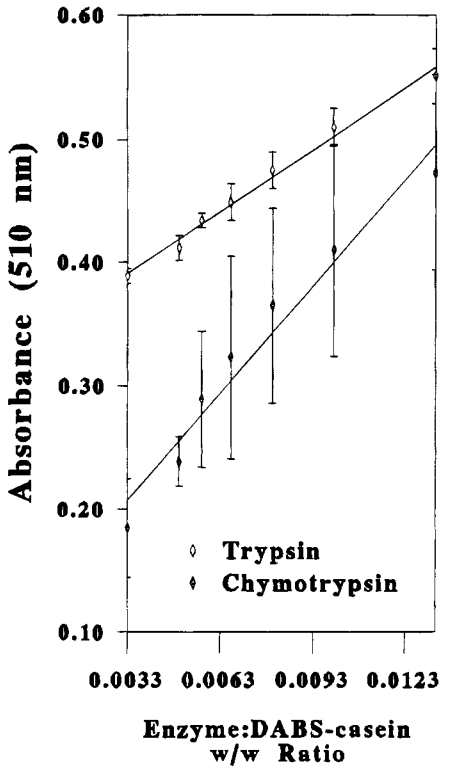

Figure 5. Effect of E:S ratios (w/w) on assay linearity. E:S ratio ranged from 1.33×10^{-2} to 3.33×10^{-3} for both trypsin and chymotrypsin.

molar ratios 2×10^{-2}–2×10^{-3} and from 10 to 120 min for E:S molar ratios 1×10^{-3}–2×10^{-4}. Regardless of the enzyme, the E:S ratio, and the incubation time ≥30 min, all of the assays had excellent linearity, $r \geq 0.9$. This indicated that an incubation time of at least 30 min would be useful in maintaining the assay linearity over a wide range of E:S ratios (r value for trypsin E:S 2×10^{-2}–5×10^{-4} was 0.904 and that for chymotrypsin E:S 2×10^{-2}–1×10^{-3} was 0.897, when incubation time was 30 min). As expected, at lower enzyme concentrations (E:S ≤ 1×10^{-3}), increasing the incubation time to 120 min afforded reliable absorbance values while maintaining the assay linearity. Additional assays with E:S ratios in the range 5×10^{-2}–1×10^{-3} and a fixed incubation time of 30 min clearly indicated that the assay was linear over a wide range of E:S ratios. When the data were analyzed between 1.33×10^{-2} and 3.33×10^{-3}, the corresponding r values for trypsin and chymotryspin were, respectively, 0.995 and 0.979 (Figure 5). The influence of incubation time on assay linearity was further evaluated at E:S ratio 2×10^{-3}. The r values for trypsin and chymotrypsin were 0.864 and 0.793, respectively, when the data were analyzed for incubation times of up to 120 min. When the data were analyzed for incubation times of up to 30 min, however, the corresponding r values were 0.969 and 0.971 (Figure 4). These data suggest an incubation time of 30 min is useful for assay linearity over a wide range of E:S ratios for both trypsin and chymotrypsin. Since the UV absorbance method is widely used for proteolytic assays, we included such assays in our study for comparative purposes under the same assay conditions. The r values for influence of time of incubation up to 30 min (under the same assay conditions as those for DABS–casein as the substrate) were 0.97 and 1.00 for trypsin and chymotrypsin, respectively. The assay linearity of DABS–casein as the substrate is therefore comparable to that of the UV absorbance method.

Assay Sensitivity. The lowest quantity of enzyme activity that can be detected by this colorimetric assay was determined. A net absorbance reading ≥0.05 on the visible spectrophotometer was considered to be the detection limit and acceptable value. Hydrolysis of DABS–casein at molar ratios between 1×10^{-3} and 2×10^{-4} was used to measure the sensitivity of the enzyme assay for both trypsin and chymotrypsin. Enzyme activity was detected at E:S ratios as low as 2.5×10^{-4} and 3.33×10^{-4} for trypsin and chymotrypsin, respectively, at 2 h of incubation (Figure 6). The r values for these assays remained >0.9. This indicated that this new method for proteolytic enzyme assay was sensitive to 27 pmol (64.16 ng) and 36 pmol (89.86 ng) for trypsin and chymotrypsin, respectively. When incubation time was at 1 h, trypsin activity could be detected at 5×10^{-4} E:S molar ratio (or 52.5 pmol enzyme). The detection limit for chymotrypsin activity at 1 h was observed at 1×10^{-3} E:S molar ratio (or 108 pmol of enzyme). For the 30 min incubation, this assay could detect 105 and 100 pmol of trypsin and chymotrypsin (250 ng each), respectively. These data indicated that this assay affords the same range of sensitiv-

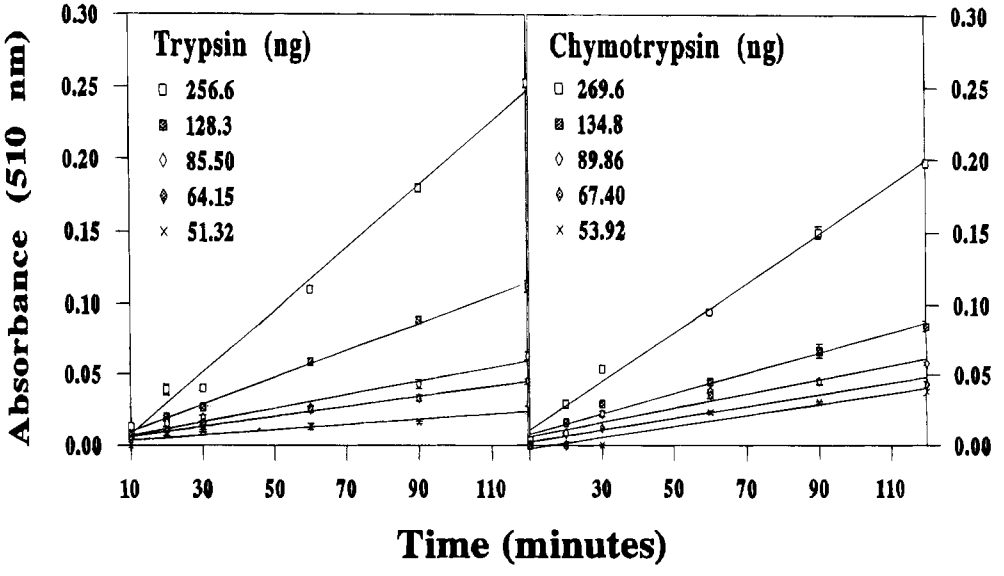

Figure 6. Influence of concentration of trypsin and chymotrypsin on assay linearity.

ity as do the radioactive and fluorescent assays, which have a sensitivity range of 5–100 ng for trypsin (Sarath, et al., 1989, and references cited therein; Twining, 1984, and references cited therein; Bolger and Checovich, 1994). This method has several advantages over the assay methods using radioactive isotopes or fluorescence labels because this method does not require specialized expensive equipment and does not require expensive disposal of radioactive waste products that are generated in methods employing radioactive isotopes.

Influence of Inhibiting Agents on the Assay. Under the assay conditions used, reducing agents (β-ME and DTT up to 40 mM in each case), a chelating agent (EDTA up to 40 mM), phenolic compounds (resorcinol, catechol, phloroglucinol, tannin up to 20 mM in each case), and detergent (SDS up to 20 mM), at their highest concentration tested, caused a small but significant decrease in the absorbance (13–22% decrease compared to the original enzyme activities) while maintaining the assay linearity ($r > 0.9$). Porter and Preston (1975) indicated that trypsin and chymotrypsin were resistant to denaturation in the presence of 35 mM SDS. Our observations, indicating that these enzymes remained active in 20 mM SDS, are consistent with those of Porter and Preston. Simple sugars (glucose, galactose, fructose, and sucrose) up to 200 mM and phytic acid (20 mM) did not decrease the absorbance significantly. Twining (1984) reported that in the presence of 200 mM sucrose the fluorescence yield of the trypsin activity was reduced in the fluorescein isothiocyanate–casein assay. On the basis of the absorbance values we obtained (range 0.4–0.65) our assay is therefore useful even in the presence of these commonly encountered chemicals in buffers and food samples. The influence of NaCl between 0 and 2 M on this assay was also evaluated. At high concentrations of NaCl (≥0.5 M) there was a marked reduction in absorbance in which the activities of both enzymes were reduced by 25–75% of the original activity. The assays did remain linear, however ($r > 0.93$). This was expected since in dilute enzyme solutions high NaCl amounts may cause some "salting out" of the enzyme protein. At all of the NaCl concentrations tested, the enzyme activities can be still measured with reliable absorbance readings.

Conclusions. DABS–casein is a suitable and stable substrate for measuring proteolytic activity. This assay procedure does not require expensive instrumentation or disposal of radioactive waste. The assay uses a visible spectrophotometer and is useful over a wide range of enzyme to substrate ratios and incubation times. The assay has sensitivity comparable to those using fluorescence and radioactive labels for such purposes. This assay can tolerate the presence of several common chemicals encountered in biological buffers and foods. Additional studies need to be done to expand the assay utility to other enzymes and food systems.

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