Physicochemical and Enzymatic Properties of Benzyl Isothiocyanate Derivatized Proteinases

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This paper deals with interactions of benzyl isothiocyanate (benzyl-ITC) with cysteine proteases (bromelain and papain) as well as with serine proteases (trypsin and α-chymotrypsin). The derivatives formed with different amounts of benzyl-ITC (10−125 mg of benzyl-ITC/g of protein) have been characterized in terms of their physicochemical and proteolytic properties. Detectable changes in the chromatogram pattern of the derivatives coupled with an increase in hydrophobicity were documented by RP-HPLC. Furthermore, the isoelectric point was shifted to the lower pH values. SDS−PAGE and MALDI-MS of the chymotrypsin derivatives showed distinctive molecular changes. The other major subject of the present paper shows the effects of benzyl-ITC derivatization on proteolytic activity of bromelain, papain, trypsin, and α-chymotrypsin. In general, a decrease of enzyme activity was documented for the proteolysis of casein and myoglobin as substrates.

Keywords: *Isothiocyanates; enzyme derivatization; bromelain; papain; trypsin; α-chymotrypsin; proteolytic degradation; casein; myoglobin; RP-HPLC; IEF; MALDI-MS*

INTRODUCTION

Benzyl glucosinolate occurs naturally in several plant species, such as Indian cress (*Tropaeolum majus* L.), garden cress (*Lepidium sativum* L.), *Carica papaya* L., and other *Carica* species (Brüsewitz et al., 1977; Mennicke et al., 1988; Bennett et al., 1997). During tissue disruption benzyl glucosinolate may come into contact with cytosolic thioglucosidases (myrosinases), resulting in the formation of breakdown products such as benzyl isothiocyante (ITC) (Bennett et al., 1997). The well-known high reactivity of ITC is largely due to its strong electrophilic nature and results in reactions with nucleophiles at physiological pH (Kroll et al., 1994a,b). One such reaction represents the conjugation with reduced glutathione in the initial stage in mercapturic acid biosynthesis for elimination of foreign compounds from the body (Brüsewitz et al., 1977). Model investigations using NMR spectroscopy showed that the reaction of benzyl-ITC takes place first at the sulfhydryl group of the reduced glutathione (Kroll and Jancke, 1994). The kinetics and equilibria of reactions of ITC with sulfide and a series of thiols, such as ethanethiol, mercaptoacetic acid, 2-mercaptoethanol, and dithiothreitol, have been reported (Drobnica and Gemeiner, 1975; Drobnica et al., 1975). The reaction of ITC with sulfhydryl groups of egg white proteins and of sarcoplasmic proteins has been indicated by determination of carbon disulfide liberation from their dithiocarbamate esters (Kroll et al., 1994a,b; Rawel and Kroll, 1995). It has been reported that the majority of ITCs hold to the generalization that their action may lie in the exclusion of key processes in the energy and intermediary cell metabolism by inhibition of some enzymes which require free sulfhydryl groups for their catalytic activity (Tang, 1974; Drobnica and Gemeiner, 1976). From this physiological point of view, the main objective of this paper was to show a reaction at the sulfhydryl group and free amino groups and to characterize the enzymes obtained after ITC derivatization with respect to changes in their physicochemical properties and proteolytic activity. Bromelain and papain (plant enzymes) were chosen, because both have a cysteine group in the active center. An another interesting reaction of ITC could be with the nucleophilic agents having OH ions as reported by Drobnica and Gemeiner (1976). Kishore Kumar Murthy and Narasinga Rao (1986) also report a possible reaction at the phenolic group of tyrosine. On the basis of these observations, the second objective was to test if a benzyl-ITC derivatization of trypsin and α-chymotrypsin (both have a serine residue in the active center) has any influence on their enzymatic properties. Benzyl-ITC was chosen because it showed the highest reactivity in previous derivatization experiments with egg white proteins (Kroll et al., 1994a).

MATERIALS AND METHODS

Investigated Proteins, Enzymes, and Derivatization with the ITCs. Hammarsten grade casein (Merck, Darmstadt, Germany), myoglobin from horse heart, lysozyme from hen egg (Fluka Chemie AG, Buchs, Switzerland), and bovine serum albumin (BSA) (Serva, Heidelberg, Germany) were the main substrates applied. The following enzymes were investigated: bromelain from pineapple stem (EC 3.4.22.32, protein content = 35.8%, Fluka Chemie AG), 2.1 units/mg of solid (1 unit will release 1.0 μmol of 4-nitrophenol/min at pH 4.6 at 25 °C with N_α-carbobenzoxyl-L-lysine-4-nitrophenyl ester as substrate); papain from papaya latex (EC 3.4.22.2, protein content = 59%, Sigma Chemical Co., St. Louis, MO), 1.7 unis/mg of solid [1 unit will hydrolyze 1.0 μmol of N_α-benzoyl-L-

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arginine ethyl ester (BAEE)/min at pH 6.2 at 25 °C]; trypsin from porcine pancreas (EC 3.4.21.4, protein content = 98%, Sigma Chemical Co.), 14 900 units/mg of solid (1 BAEE unit = ΔA_{253} of 0.001 per minute with BAEE as substrate at pH 7.6 at 25 °C); α-chymotrypsin from bovine pancreas (EC 3.4.21.1, protein content = 96%, Fluka Chemie AG), 53.1 units/mg [1 unit will hydrolyze 1 μmol of Suc-$(ala)_2$-pro-phe-4-NA/min at pH 7.8 and 25 °C]. All specific activities and definitions quoted here were given by the producers.

Crude powder (8.475 g) from papaya latex containing papain was dispersed in water, pH value adjusted to 9 using 0.5 M NaOH, and filtered, and the clear solution was made up to 200 mL and divided into five parts (40 mL) each containing 1 g of protein. These solutions were allowed to react with different amounts of benzyl-ITC (10, 25, 62.5, and 125 mg of benzyl-ITC in 10 mL of ethanol) under continuous stirring for 3 h. To the control sample was added only 10 mL of ethanol. The procedure was repeated three times, and the respective products were pooled, dialyzed against deionized water for 18 h, and lyophilized. The dried samples contained between 70 and 85% protein as determined by semimicro-Kjeldahl analysis (Kjeldatherm System KT 40, Gerhardt Laboratory Instruments, Bonn, Germany).

Crude bromelain (a mixture of cysteine proteinases with stem bromelain as the major component) was applied directly without filtration as commercially obtained. The derivatization (25, 62.5, and 125 mg of benzyl-ITC/g of protein) was carried out as described above for papain. The protein content of the lyophilized control and benzyl-ITC-derivatized bromelain ranged between 61.4 and 66.8% as determined by Kjeldahl analysis.

Trypsin (1 g in 40 mL of 0.1 M Tris-HCl, pH 8) was derivatized with different amounts of benzyl-ITC (10, 25, 62.5, and 125 mg of benzyl-ITC/g of protein, each in 10 mL of ethanol) under continuous stirring at 4 °C for 6 h. The product thus obtained was dialyzed against 0.001 M HCl at 4 °C for 18 h and lyophilized.

The chymotrypsin derivatives were prepared in a similar way to trypsin with different amounts of benzyl-ITC (10, 25, 62.5, and 125 mg of benzyl-ITC/g of protein) but under continuous stirring at 4 °C for 24 h.

Physicochemical Characterization. Determination of free amino groups was carried out according to the method of Adler-Nissen (1972) using trinitrobenzenesulfonic acid (TNBS) in a 1% sodium dodecyl sulfate (SDS) solution of the samples. The reaction at sulfhydryl groups was monitored as done methodically for fungicides (Engst and Schnaak, 1974). The principle of this method involves the decomposition of dithiocarbamates followed by absorption of the released carbon disulfide in ethanolic copper acetate/diethanolamine solution and finally the photometric determination of the complex copper dithiocarbamate. The free SH groups were determined according to the method of Hoffmann (1977) using dithiobis-(2,4-nitrobenzoic acid) (DTNB) reagent.

The solubility profile of the lyophilized samples under various pH conditions was determined in a 0.05 M sodium phosphate/citric acid buffer system by removing the insoluble material through centrifugation at 10 700 rpm (9088*g*, 10 min, Megafuge 2.0R, Heraeus, Hanau, Germany).

The protein content in the solutions was determined according to a modified Lowry method (Lowry et al., 1951; Markwell et al., 1978). The control protein preparation was used in each case to calibrate the regression curves (bromelain, $Y = 0.0523 + 0.0182X$, $R^2 = 0.99$; papain, $Y = 0.0588 + 0.00651X$, $R^2 = 0.95$; trypsin, $Y = 0.0859 + 0.0151X$, $R^2 = 0.99$; chymotrypsin, $Y = 0.127 + 0.00717X$, $R^2 = 0.99$) after its protein content was determined by semimicro-Kjeldahl analysis (Kjeldatherm System KT 40, Gerhardt Laboratory Instruments).

The changes in hydrophobic/hydrophilic character of the benzyl-ITC-derivatized enzymes were investigated by RP-HPLC, which was conducted with a Jasco (Gross-Umstadt, Germany; Tokyo, Japan) chromatographic system using a 20−54% acetonitrile gradient (both eluents acidified with HCl, 1 mL of concentrated HCl/1000 mL of eluent; gradient: 5 min, 100% A; 20 min, 0−100% B; 15 min, 100% B; 5 min, 0−100% A; 10 min, 100%A) on a Grom-Sil 300 RP-C18 column (250 × 4 mm; 300 Å; 5 μm, UV detection at 220 nm) at 25 °C. The surface hydrophobicity of the samples was also determined for benzyl-ITC-derivatized chymotrypsin using a hydrophobic fluorescence probe, 1-anilino-8-naphthalensulfonate (ANS), according to the method of Hayakawa and Nakai (1985). The initial slope (S_0) of the fluorescence intensity versus soluble protein concentration plot was used as an index of the protein hydrophobicity.

Isoelectric focusing on polyacrylamide gel (8% T, Bio-Rad apparatus, Munich, Germany) was conducted as described in Kroll and Rawel (1996). Servalyte pH 3−10 (Serva, Heidelberg, Germany) was applied as carrier ampholyte, and the samples were generally applied on the cationic side. Sodium hydroxide and phosphoric acid were used as cathode and anode buffers, respectively. Pre-electrophoresis was done at 600 V with 5−15 mA for 1 h. The main electrofocusing was conducted at 600 V and 15 mA for 3−4 h. The proteins were fixed in gel with trichloroacetic acid, and Coomassie brillliant blue R 250 (Serva, Heidelberg, Germany) was used to dye the proteins. SDS−PAGE according to the method of Laemmli (1970) was applied for molecular weight determination. Band intensity was quantified using a computerized elscript 400 densitometer (Hirschmann Gerätebau, Taufkirchen, Germany) according to the manufacturer's instructions.

MALDI-TOF mass spectrum analysis was performed on a Bruker REFLEX II (Bruker Franzen Analytik, Bremen, Germany) instrument equipped with a reflector and delayed extraction (Vestal et al., 1995). The intact molecular weights were measured with DHBs (Rosinke et al., 1995), a binary matrix consisting of 9 parts of 2,5-dihydroxybenzoic acid and 1 part of 2-hydroxy-5-methoxybenzoic acid, both solutions 25 g/L in 35% acetonitrile/65% 0.1% TFA. The samples were prepared by mixing 1 μL of matrix and 0.2 μL of sample on the target and letting it air-dry. The spectra were externally calibrated on myoglobin, singly and doubly charged peaks.

Characterization of the Enzyme Activities. The influence of benzyl-ITC derivatization on proteolytic properties of bromelain, papain, trypsin, and chymotrypsin was demonstrated by its action on casein according to the assay method by Murachi (1970). Casein (3 g) was dissolved in 250 mL of 0.03 M potassium phosphate buffer, pH 7.5, by heating in a boiling bath for 15 min. The final pH was 7.2. Freshly prepared L-Cys (0.15 M) was used to activate bromelain and papain (omitted during casein degradation with trypsin and chymotrypsin). TCA solution contained 18 g of TCA, 30 g of sodium acetate, and 39 mL of glacial acetic acid filled with water to a final volume of 1000 mL. Detailed assay procedure for caseinolytic activity is described in Murachi (1970). The analysis was modified slightly by measuring at 280 nm instead of 275 nm with a dilution step (1:4) before measurement of the absorption. The centrifugation step to separate TCA soluble peptides was adjusted to 9088*g* for 10 min (Heraeus, Megafuge 2.0R, Hanau, Germany). Trypsin−benzyl-ITC derivatives (protein content = 98%) in enzyme/substrate ratio (E/S, w/w) = 1:30 and bromelain−benzyl-ITC derivatives (protein content = 61.4−66.8%) in E/S = 1:60 (w/w) were applied for casein hydrolysis. In the case of papain−benzyl-ITC-derivatized samples (protein content = 70−85%) and chymotrypsin−benzyl-ITC-derivatized samples (protein content = 95−97%), the extent of casein hydrolysis (E/S = 1:60, w/w) was monitored by RP-HPLC of the TCA soluble peptides on a Micra-NPS-C18 column (33 × 4.6 mm, 1.5 μm, Northbrook, IL; flow rate = 0.5 mL/min; UV detection at 220 nm) with a column temperature of 25 °C using a Jasco chromatographic system. A distilled water/acetonitrile (both eluents acidified with 0.1% trifluoracetic acid, v/v) gradient was applied under the following conditions: 100% water, 2 min; 0−70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 0−100% water, 4 min; 100% water, 10 min (equilibration). The injection volume of the samples was 5 μL. The total peak area of the peptides was used to quantify the extent of tryptic digestion. The actual specific activity with enzyme with regard

Table 1. Characterization of ITC-Derivatized Enzymes Depending on the Degree of Derivatization: Free Amino Groups and Reaction at the SH Groups[a]

enzyme	control	10 mg of benzyl-ITC/g of protein	25 mg of benzyl-ITC/g of protein	62.5 mg of benzyl-ITC/g of protein	125 mg of benzyl-ITC/g of protein
bromelain					
CS_2 content (μg/g)	0		0	14	41
SH groups (nmol/mg)	6.9 ± 0.5		5.1 ± 0.3	5.2 ± 0.2	5.3 ± 0.0
NH_2 groups (nmol/mg)	753 ± 3		657 ± 8	591 ± 8	559 ± 9
papain					
CS_2 content (μg/g)	0	0	0	0	116
SH groups (nmol/mg)	1.1 ± 0.11	1.4 ± 0.04	1.8 ± 0.15	1.2 ± 0.25	2.6 ± 0.15
NH_2 groups (nmol/mg)	2131 ± 3	2057 ± 6	1973 ± 6	1894 ± 13	1298 ± 4
chymotrypsin					
SH groups (nmol/mg)	0	0	0.20 ± 0.03	0.56 ± 0.05	0.61 ± 0.04
NH_2 groups (nmol/mg)	627 ± 5	597 ± 10	707 ± 3	705 ± 4	701 ± 5
trypsin					
SH groups (nmol/mg)	0	0	0	0	0
NH_2 groups (nmol/mg)	376 ± 4	372 ± 5	360 ± 9	355 ± 7	347 ± 9

[a] Indirectly estimated as released CS_2 and with DTNB method.

to enzyme units for the ITC derivatives and controls of the respective enzymes was not determined, because the objective was to show the trend in the change of caseinolytic activities.

Myoglobin was generally dissolved in 8 M urea/buffer system, heated at 100 °C for 5 min, cooled and the reaction mixture volume diluted to give final urea concentration of 2 M. Hydrolysis of myoglobin with bromelain-benzyl-ITC derivatives and control (E:S = 1:40, 0.15 mg bromelain-protein/6 mg myoglobin in 1.6 mL buffer) was performed under activation with cysteine (0.15 M) in 0.2 M sodium phosphate−citrate buffer pH 7.6 containing 2 M urea at 35 °C. After different lengths of time (5, 24 min), a sample was directly injected on MICRA-NPS-C18 column and RP-HPLC under conditions described above was performed. The peak area of the undigested myoglobin in comparison to peak area before digestion (100%, without enzyme) was used to determine percent hydrolyzed myoglobin.

Proteolysis of myoglobin with papain (activation with cysteine, E/S = 1:40, 0.15 mg of papain protein/6 mg of myoglobin in 1.6 mL) and chymotryptic hydrolysis of myoglobin (E/S = 1:60, 0.1 mg of chymotrypsin/6 mg of myoglobin in 1.6 mL) was investigated by incubation at 35 °C (2 M urea, 0.1 M Tris-HCl, pH 8, 0.02 $CaCl_2$). After different lengths of digestion, the reaction was stopped by addition of an equivalent aliquot volume of TCA (20% TCA). The reaction mixtures were allowed to stand for 10 min and then centrifuged (9088*g*, 10 min, Heraeus, Megafuge 2.0R). RP-HPLC of the TCA soluble peptides using a 0−70% acetonitrile/water gradient was performed on a Micra-NPS-C18 column as described above. The total peak area of the peptides was used to quantify the extent of digestion with papain and chymotrypsin.

Similarly, the tryptic hydrolysis of myoglobin (E/S = 1:40), lysozyme (E/S = 1:40), and BSA (E/S = 1:80) was carried out in 0.1 M Tris-HCl, pH 8, containing 0.01 M $CaCl_2$ and 2 M urea at 35 °C. All proteins were denatured in 8 M urea/buffer system at 100 °C for 3−5 min and diluted before being applied. The liberated TCA soluble peptides were analyzed using a modified Lowry procedure and expressed in micrograms (Lowry et al., 1951; Markwell et al., 1978). A solution of the respective protein was used for blind analysis, where the enzyme solution was replaced by the buffer system, incubated under the same conditions. The TCA soluble part of this solution was used as blank in the Lowry procedure. The Lowry regression standard curve for each substrate protein as reference (myoglobin, $Y = 0.0888 + 0.00719X$, $R^2 = 0.996$; lysozyme, $Y = 0.1125 + 0.00610X$, $R^2 = 0.99$; BSA, $Y = 0.03670 + 0.00616X$, $R^2 = 0.98$) was applied to calculate the amount of peptides released in respective cases.

Statistical Analysis. The digestions and analysis were repeated three times and analyzed by means of standard deviation (SD). A maximum of ±5% SD from the averaged values was generally observed. The averaged values are evaluated in the respective figures.

RESULTS AND DISCUSSION

Characterization of Physicochemical Properties. *Reaction at the Sulfhydryl Groups.* Isothiocyanates react with the deprotonated SH groups of proteins to give the esters of dithiocarbamic acids as already described in Kroll et al. (1994a,b). The reaction at the SH groups can be followed by liberation of CS_2 from the dithiocarbamic acid esters as already described in our previous papers (Kroll et al., 1994a,b). Bromelain and papain both have only one reactive cysteinyl residue per molecule located in the active center of the enzymes, and their structural conformation is stabilized by three disulfide bridges (Arnon, 1970; Murachi, 1970). In the case of papain it has been documented that a complete rupture of the disulfide bridges may lead to loss of biological, catalytic, and immunological activity (Arnon, 1970). A cleavage of disulfide may take place during reaction with ITC as reported for lysozyme in Kroll et al. (1994b). The amount of CS_2 liberated from bromelain derivatives increased with higher amounts of ITC added (62.5 and 125 mg of ITC/g of protein; Table 1). The amount of SH groups as identified by DTNB reagent in ITC-derivatized bromelain decreased in the first instance by the addition of 25 mg of benzyl-ITC and then remained more or less constant when higher amounts of ITC were used for derivatization (Table 1). This means that ITC causes a splitting of disulfide bonds, followed by a reaction with the free sulfhydryl and finally displacement by DTNB. In the case of papain, only the sample prepared with the highest amount of benzyl-ITC (125 mg/g of protein) showed the presence of dithiocarbamic acid ester and a slight increase in the amount of SH groups as determined using DTNB reagent (Table 1). α-Chymotrypsin is known to contain five disulfide bridges, two of which link three polypeptide chains (GenBank protein sequences, Accession No. 67573; Lehninger et al., 1994). Because limited amounts of the ITC-derivatized chymotrypsin and trypsin samples were available, it was not possible to perform the estimation of carbon disulfide liberation. In this case only the amount of free sulfhydryl groups was determined using the DTNB method (Hoffmann, 1977). The results showed a very slight increase in the amount of free sulfhydryl groups with increasing amount of reacting benzyl-ITC (from being practically "not detectable" in the control sample to 0.6 nmol/mg in the chymotrypsin sample derivatized with 125 mg of benzyl-ITC/g of protein; Table 1). This

indicates that a rupture of disulfide bridges has taken place. No sulfhydryl groups were found in trypsin with DTNB analysis.

Reaction at the Free Amino Groups. The isothiocyanates also react with the free amino groups, preferentially in the alkaline pH region, to form thiourea derivatives (Kishore Kumar Murthy and Narasinga Rao, 1986). The amount of free amino groups decreased with increasing degree of derivatization (except chymotrypsin) as shown in Table 1. Theoretically, bromelain has ~701 nmol of amino groups/mg [calculated using amino acid sequence according to Ritonja et al. (1989)]. We estimated 753 nmol in the control sample (Table 1). This slight increase may be due to the autolysis products confirmed by SDS−PAGE (Supporting Information). The value determined for papain (2131 nmol of free amino groups/mg of protein) is much higher than the expected value of ~524 nmol/mg of protein according to Arnon (1970). This may be due to a number of small peptides produced by autolysis as confirmed using SDS−PAGE (Supporting Information). Theoretically, α-chymotrypsin contains, calculated from its sequence, ~586 nmol/mg free amino groups (GenBank protein sequences, Accession No. 67573). The value determined as per TNBS method was 625 nmol/mg of protein, slightly higher than the expected value (Table 1). Initially, in the presence of 10 mg of benzyl-ITC/g of protein, there was a decrease in the content of free amino groups, which increased over the control value in the presence of higher amounts of benzyl-ITC and remained then constant, which can be explained only by autolysis processes. Trypsin has theoretically ~458 nmol of free amino groups/mg of protein compared to the determined value of 380 nmol/mg of protein (Table 1). SDS−PAGE of the control sample showed ~90% with molecular mass of 23 kDa [23.4 kDa, according to Walsh (1970)]. The presence of a dimer during SDS−PAGE under reducing conditions (~10%, 45 kDa) could explain the relative decreased amount of free amino groups obtained (Supporting Information).

Changes in Solubility. The solubility profiles of the ITC derivatives of bromelain and papain are illustrated in Figure 1. A decrease in protein solubility with increasing amount of added ITC, especially in the pH range 3−9, was noted for both enzymes. This is caused by the decrease in the amount of the charged protein side chains after ITC derivatization. No changes of the solubility profiles of trypsin− and chymotrypsin−ITC derivatives were documented (Supporting Information), reflecting only the slight changes in the amount of free amino groups also observed (Table 1).

Changes in Hydrophile/Hydrophobe Character. The reaction of benzyl-ITC with the proteolytic enzymes produces detectable alternations in the chromatogram pattern as detected by RP-HPLC (papain, Figure 2A; chymotrypsin, Figure 2B). While increasing the amount of reacting benzyl-ITC, an increase in the retention time of the main peak was noted, documenting the respective parallel increase in the hydrophobicity. The resolution of the peaks seems also to be adversely affected, indicating possible denaturation and molecular interactions. The increase in retention time is more distinct for papain ITC derivatives (Figure 2A). ITC-derivatized bromelain showed behavior similar to that of papain and chymotrypsin, whereas trypsin ITC derivatives showed no change in their chromatographical properties, confirming the slight changes noted for their other physi-

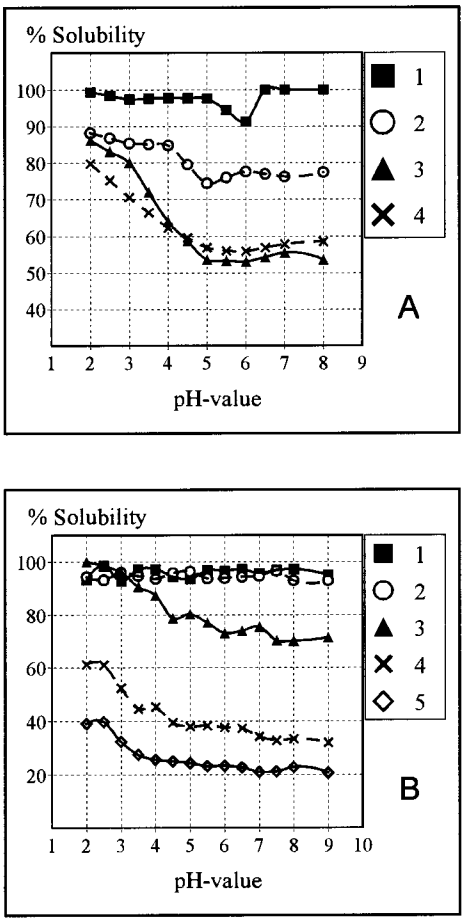

Figure 1. Effect of ITC derivatization on solubility of enzymes: (A) bromelain, (1) bromelain control, (2) 25 mg of benzyl-ITC/g of bromelain, (3) 62.5 mg of benzyl-ITC/g of bromelain, (4) 125 mg of benzyl-ITC/g of bromelain; (B) papain, (1) papain control, (2) 10 mg of benzyl-ITC/g of papain, (3) 25 mg of benzyl-ITC/g of papain, (4) 62.5 mg of benzyl-ITC/g of papain, (5) 125 mg of benzyl-ITC/g of papain.

cochemical properties as discussed above (Supporting Information). Further experiments were also conducted to measure the change in the nature of the surface hydrophobicity of chymotrypsin molecules after reaction with benzyl-ITC (due to the good solubility of its derivatives) using the fluorescence probe ANS. The results show a similar behavior of the derivatives as noted for the RP-HPLC, and a general increase in the hydrophobic nature of the derivatives compared to the control sample can be documented (Figure 3).

Isoelectric Focusing. The ITC derivatization changes the amount of charged groups as documented for free amino and SH groups (Table 1). Generally, a loss of charged groups due to nucleophilic addition of benzyl-ITC, for example, to the ϵ-amino groups of lysine is accompanied with a change of the isoelectric point of the proteins as shown for myoglobin (Kroll and Rawel, 1996). A similar behavior with regard to change in the isoelectric points of the investigated enzymes is shown for bromelain and chymotrypsin (Figure 4). The isoelectric points of bromelain components were reported to be >9.6 (Cooreman et al., 1976; Murachi, 1970). This is in agreement with the results obtained by us as shown in Figure 4A, where most of the protein fractions have isoelectric points in the pH range 8−10.5. The ITC derivatization produces protein bands with distinct isoelectric points in the pH range 6−9, showing a shift

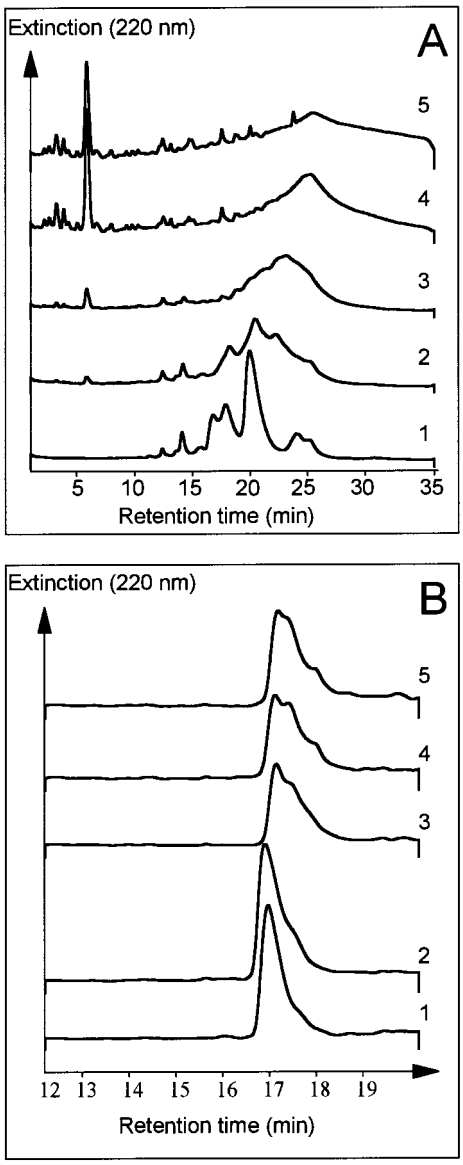

Figure 2. RP-HPLC of ITC-derivatized papain and chymotrypsin: (A) papain samples; (B) chymotrypsin samples; (1) control, (2) 10 mg of benzyl-ITC/g of protein, (3) 25 mg of benzyl-ITC/g of protein, (4) 62.5 mg of benzyl-ITC/g of protein, (5) 125 mg of benzyl-ITC/g of protein.

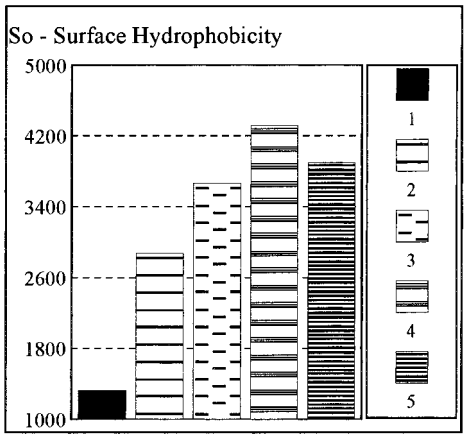

Figure 3. Change in ANS surface hydrophobicity of benzyl-ITC-derivatized chymotrypsin: (1−5) see legend to Figure 2.

of the isoelectric points in the direction of acidic pH values with increasing amounts of ITC reacted. Papain has an isoelectric point of 8.75 as reported by Arnon (1970). Our IEF results showed fractions having isoelectric points in the pH range 7.6−8.9, in agreement with this documentation. The ITC derivatization produced seven distinct protein bands in the pH range 6.1−8.2, with the highest derivatized product (125 mg of benzyl-ITC/g of protein) confirming the trend observed for bromelain samples (Supporting Information). The isoelectric point of chymotrypsin A zymogen has been reported to be at pH 9.6 (Patrickios and Yamasaki, 1995). The investigated α-chymotrypsin sample contained a series of fractions with different isoelectric points, whereby the main fraction had an isoelectric point of 7.6 (Figure 4B). The derivatization with increasing amounts of benzyl-ITC caused the isoelectric point of the main fraction to shift to lower values between 6.5 and 7.5, accompanied by formation of new electrophoretic bands (Figure 4B). Furthermore, the fractions having their isoelectric points in the acidic pH range (pH 4−5) were hardly detectable after ITC derivatization.

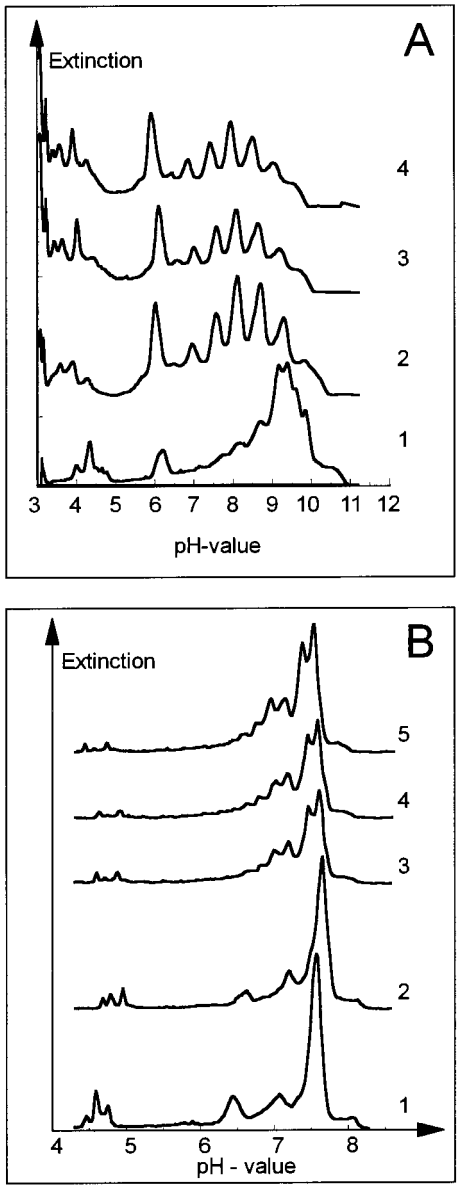

Figure 4. IEF of ITC-derivatized bromelain and chymotrypsin: (A) see legend to Figure 1; (B) chymotrypsin; (1−5) see legend to Figure 2.

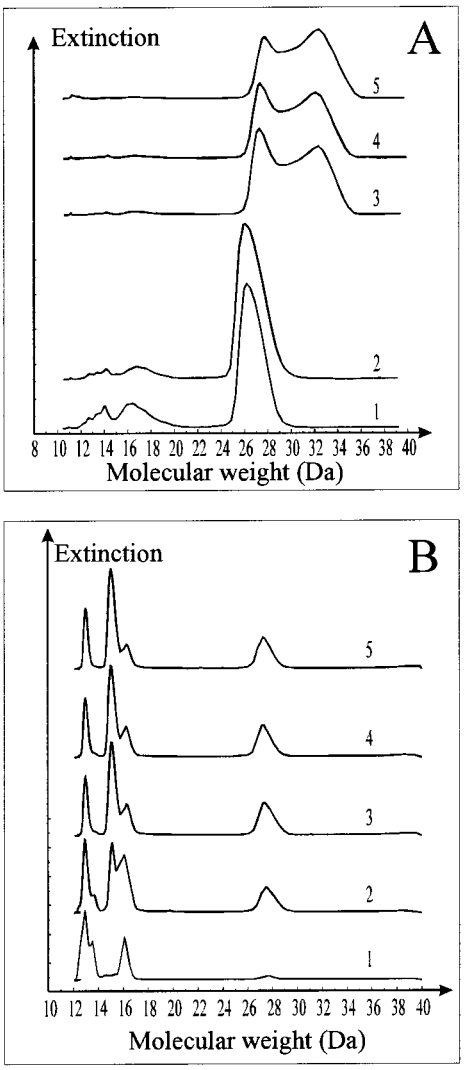

Figure 5. SDS−PAGE of benzyl-ITC-derivatized chymotrypsin: (A) under nonreducing conditions; (B) under reducing conditions; (1−5) see legend to Figure 2.

In the case of trypsin, in agreement with the low changes for the free amino groups, only slight changes in the IEF pattern were obtained after ITC derivatization (Supporting Information). The experimentally determined isoelectric point of ~9 [reported isoelectric point of 10.8 (Walsh, 1970)] underscores the result of the low amount of free amino groups determined for the control trypsin (376 nmol/mg of protein compared to the expected 458 nmol/mg of protein).

SDS−PAGE Analysis. No major changes in the SDS−PAGE electropherograms of benzyl-ITC-derivatized bromelain, papain, and trypsin were observed in comparison to their respective controls (Supporting Information). Chymotrypsin derivatives showed that under nonreducing conditions of SDS−PAGE, with increasing amounts of benzyl-ITC added during the reaction, an increase in the molecular mass range for the main fraction from 24 000−28 000 to 26 000−35 000 Da can be noted (Figure 5A). The reported molecular mass of α-chymotrypsin is 25 000 Da, in agreement with the results shown in Figure 5A (GenBank protein sequences, Accession No. 67573; Lehninger et al., 1994). SDS−PAGE under reducing conditions (Figure 5B) shows that the control chymotrypsin can be reduced to three subfractions with molecular masses in the range of 12 000−17 000 Da. The derivatization with benzyl-

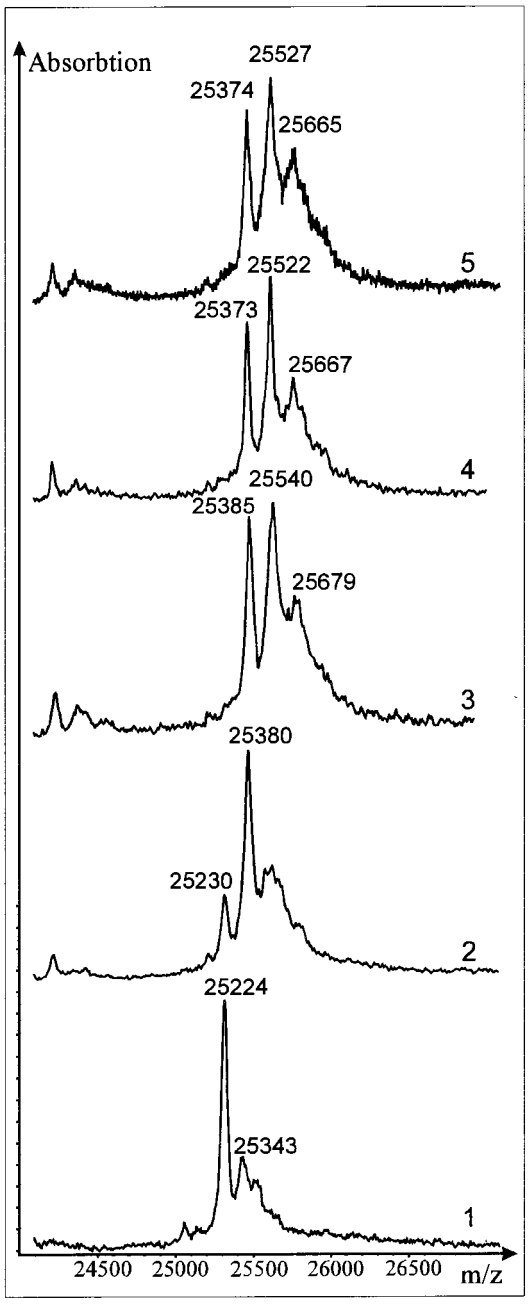

Figure 6. MALDI-MS of benzyl-ITC-derivatized chymotrypsin: (1−5) see legend to Figure 2.

ITC leads to formation of a fraction with molecular mass of 27 000 Da. Because during the sample preparation both SDS and a reducing agent (β-mercaptoethanol, denaturing conditions, 100 °C, 5 min) were applied, it can be expected that this product is held together by covalent forces other than disulfide bridges. Furthermore, it can be noted that the composition of the subfractions with molecular masses in the range of 12 000−17 000 Da also seems to be significantly affected. So, for example, the subfraction with molecular mass of 16 000 Da decreases with a simultaneous increase of the 15 000 Da subfraction (Figure 5B), which in turn is dependent on the amount of benzyl-ITC applied during the derivatization.

MALDI-MS. This method of analysis was applied as an exemplar to demonstrate detailed molecular changes in benzyl-ITC-derivatized chymotrypsin. The MALDI mass spectra of the samples obtained from the reaction

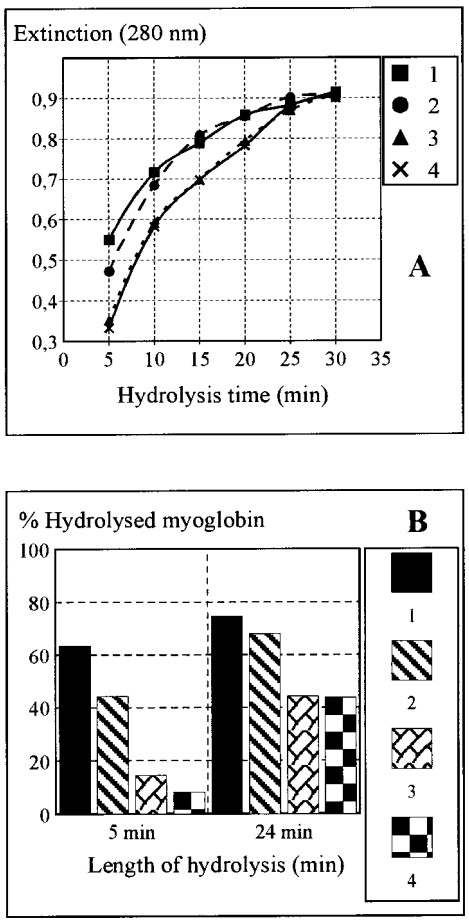

Figure 7. Effect of ITC derivatization on bromelain activity: (A) digestion of casein, E/S = 1:60; (B) digestion of myoglobin, E/S = 1:40; (1−5) see legend to Figure 1A.

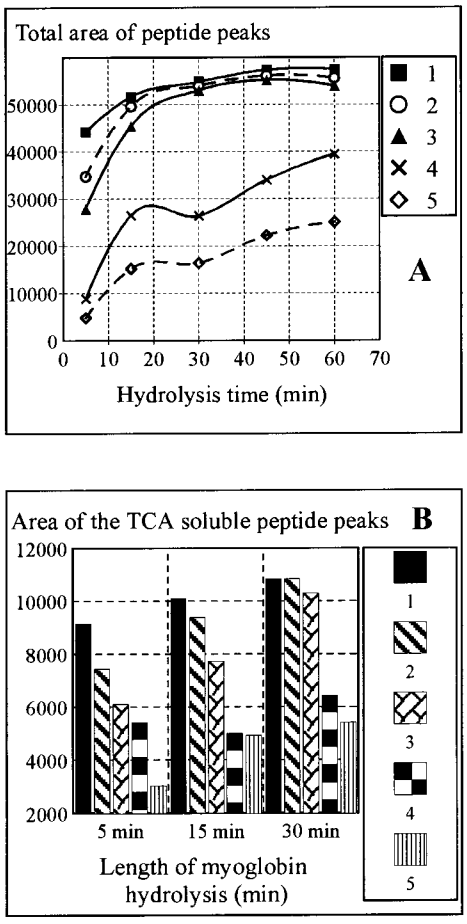

Figure 8. Effect of ITC derivatization on papain activity: (A) digestion of casein, E/S = 1:60; (B) digestion of myoglobin, E/S = 1:40; (1−5) see legend to Figure 2.

of various amounts of benzyl-ITC with chymotrypsin (between 10 and 125 mg/g of protein) are shown in Figure 6. The molecular mass of α-chymotrypsin determined according to this method was 22 524 Da, agreeing with values reported in the literature [25 000 Da (Lehninger et al., 1994)] and as determined with SDS−PAGE (Figure 5A). Each spectrum shows a mixture of products with different degrees of derivatization. The peaks are separated by an increment of ∼149 DA, accounting for addition of one molecule of benzyl-ITC. Already in the presence of 10 mg of benzyl-ITC/g of protein during the reaction with chymotrypsin, most of the enzyme has been derivatized at a single reaction site (Figure 6, curve 2). The maximum derivatization was achieved by addition of 125 mg of benzyl-ITC, which caused a derivatization of three reaction sites of the chymotrypsin molecules (Figure 6, curve 5). Because the method does not allow identification of the position of the reacting site, a peak of a distinct molecular mass certainly represents several isomers. Further experiments coupled with enzymatic hydrolysis are planned for identification of these reaction sites.

Effect on Proteolytic Properties. Looking at the reactions of ITC with proteins from a toxicological or physiological point of view, it is of importance to estimate their impact on biological and functional properties of the proteins (enzymes). With regard to this, it has been reported that the inhibition of glycolysis in yeasts, bacteria, and animal cells may be the result of inactivation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hexokinase as well as other enzymes that require free SH groups for their activity (Tang, 1974; Drobnica et al., 1975). The inhibition of some mitochondrial functions of isolated mitochondria as well as the inhibition of proteosynthesis in a cell-free system, for example, inactivation of ribosomes and some elongation factors, has also been attributed to reactions of SH groups with ITC (Drobnica et al., 1975). Reactions of benzyl-ITC with bovine sarcoplasmic proteins (especially glycolytic enzymes, e.g., kinase) also reacted preferentially with amino groups and sulfhydryl side chains to give thiourea and dithiocarbamate derivatives (Rawel and Kroll, 1995). Thus, the effects on enzymatic activity of bromelain, papain, trypsin, and chymotrypsin were investigated to gain more information on the behavior of proteolytic enzymes. Figure 7 shows that with increasing amounts of benzyl-ITC derivatization of bromelain, a decrease in the amount of hydrolyzed casein and myoglobin was observed. If we compare this decrease in the relative enzyme activity with the results shown in Table 1, a regression coefficient of 0.70 can be determined for the decrease in hydrolysis of casein (after 10 min) and myoglobin (after 5 min) against the amount of dithiocarbamic acid ester formed (estimated by the release of CS_2). However, in this connection, the increase in the amount of blocked amino groups of bromelain as a result of the reaction with benzyl-ITC correlated much better with the respective decrease in enzyme activity (correlation factor = 0.91, casein, 10 min hydrolysis; for myoglobin = 0.97; 5 min hydrolysis). This means that the reaction with the free amino groups and the resulting structural

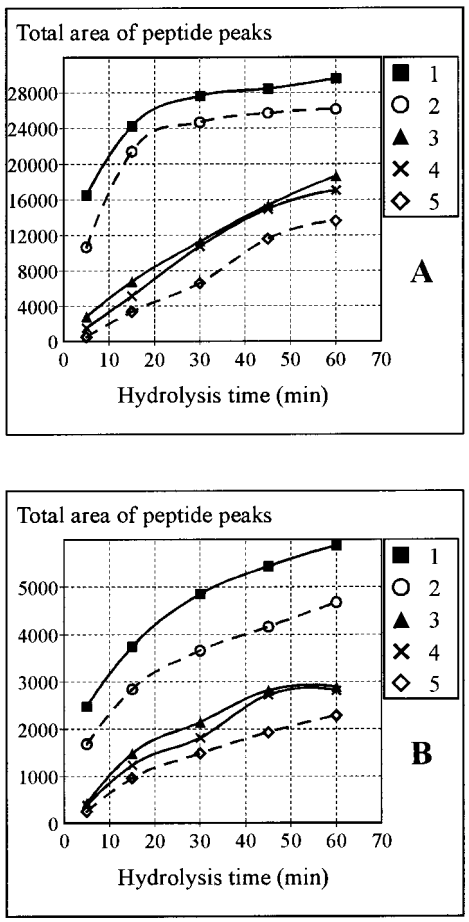

Figure 9. Effect of ITC derivatization on chymotrypsin activity: (A) digestion of casein, E/S = 1:60; (B) digestion of myoglobin, E/S = 1:60; (1−5) see legend to Figure 6.

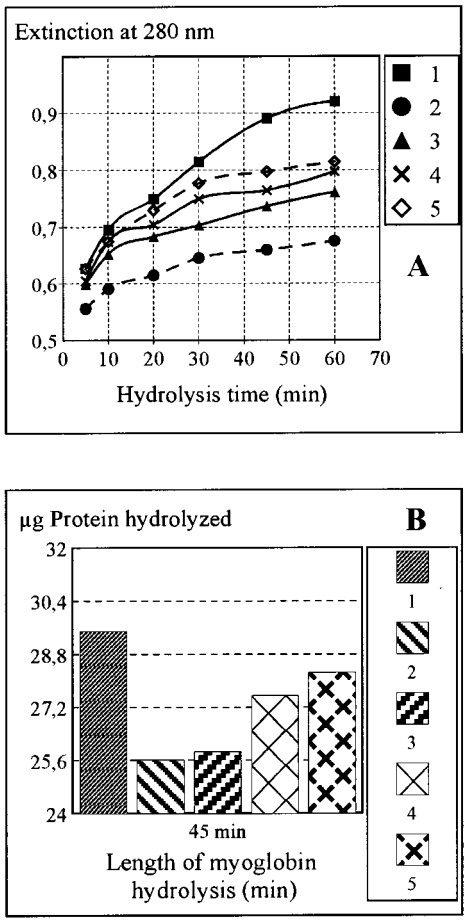

Figure 10. Effect of ITC derivatization on trypsin activity: (A) digestion of casein, E/S = 1:30; (B) digestion of myoglobin, E/S = 1:40; (1−5) see legend to Figure 2.

changes are of primary importance in decreasing the bromelain activity, and these reactions precede the reaction at the SH groups (see also Table 1). In the case of papain, a decrease in proteolytic activity against the substrates casein and myoglobin was also observed with respect to an increase in the amount of ITC present during derivatization (Figure 8). A significant correlation (0.84) was obtained by plotting the decrease in myoglobin hydrolysis (after 5 min) against the increasing amount of blocked free amino groups (Table 1). As observed for bromelain, the ITC derivatization of the free amino groups was better correlated with decreasing papain activity than with the reacted SH groups. As a consequence of these reactions, the highest degree of benzyl-ITC derivatization (125 mg of ITC/g of protein; highest amount of amino and SH groups blocked) also produced a papain product with the lowest proteolytic activity. Similar results indicating an inhibition of papain were also reported by Tang (1974), whereas using a low molecular mass substrate (*N*-carbobenzoxyglycine-*p*-nitrophenyl ester). Tang (1974) further reported a restoration of papain activity by cysteine, which was not observed during our investigations.

The effect of the reaction of benzyl-ITC with chymotrypsin on its proteolytic properties has been documented in Figure 9 using casein and myoglobin as substrates. In both cases a distinct decrease in the enzyme activity was witnessed. It could be further shown that the decrease in enzyme activity was dependent on the amount of benzyl-ITC present in the reaction mixture, although a derivatization above 25 mg of benzyl-ITC/g of protein did not produce a much higher change regarding this decrease of the proteolytic activity.

In contrast to the results observed for bromelain, papain, and chymotrypsin, the proteolytic activity of trypsin was most affected by the smallest amount of benzyl-ITC present during derivatization (10 mg of benzyl-ITC/g of protein, Figure 10). The influence decreased with increased amount of benzyl-ITC added. A similar trend in the decrease of tryptic activity of ITC-derivatized samples was also noted for other substrates (lysozyme and BSA, Supporting Information). At this stage the following explanation can be proposed: The conformational changes in the presence of a high concentration of hydrophobic benzyl-ITC make the reactive groups in the active center of trypsin, contrary to the other investigated enzymes, inaccessible to the reagent. Because the reaction at the free amino groups is negligible (especially for derivative with 10 mg of benzyl-ITC/g of protein; Table 1), it is likely that a reaction at the serine side chain in the active center of trypsin might have taken place. Kawakishi and Kaneko (1987) reported that the trypsin activity was inhibited by free allyl-ITC. However, because allyl-ITC is unstable in aqueous solution and decomposes easily, they assumed the possibility of an inhibitory effect of allyl-ITC to bevery low. On the other hand, Drobnica et al. (1975) report possible reactions of ITC with −OH groups, confirming the possibility of the above-mentioned reaction. As a result of these preliminary

observations, these atypical behaviors of ITC-derivatized trypsin remain a subject of our further studies.

In conclusion, the reactions of ITC with proteolytic enzymes (bromelain, papain, chymotrypsin, and trypsin) induce changes in physicochemical and enzymatical properties. There is a decrease in the amount of free amino groups, and a reaction at the sulfhydryl group takes place. Further investigations are needed to confirm the reaction of ITC at OH groups of proteins. Chromatographical and electrophoretical behavior as shown by RP-HPLC and IEF was changed. The investigated enzymes showed a lower enzyme activity after derivatization during proteolysis of casein and myoglobin as substrates. These results are important with regard to further experiments planned involving physiological and toxicological effects of ITC-derivatized proteins.

ABBREVIATIONS USED

ANS, 1-anilino-8-naphthalensulfonate; BAEE, N_α-benzoyl-L-arginine ethyl ester; BSA, bovine serum albumin; DTNB, dithiobis(2,4-nitrobenzoic acid); ITC, isothiocyanate; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SD, standard deviation from the average value; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TNBS, trinitrobenzenesulfonic acid.

Supporting Information Available: Results of SDS−PAGE, solubility profiles, RP-HPLC results, IEF of papain, IEF of trypsin, and results of tryptic hydrolysis of lysozyme and bovine serum albumin (8 pages). Ordering information is given on any current masthead page.

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