Reactions of Allyl Isothiocyanate with Alanine, Glycine, and Several Peptides in Model Systems

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The nucleophilic addition reactions of allyl isothiocyanate (AITC) with alanine, glycine, and five alanine and/or glycine containing di- and tripeptides were investigated in model aqueous solutions of pH 6, 8, and 10 at 25 °C for 2−4 weeks. The formation of primary adducts, i.e., *N*-allylthiocarbamoyl amino acids (ATC-amino acids) or ATC-peptides, their transformation products, i.e., 3-allyl-2-thiohydantoins originating by cyclization of ATC-amino acids or by cleavage of ATC-peptides, and several other minor components were observed. The results revealed that both addition and cleavage rates rise proportionally to pH, whereas the formation of 2-thiohydantoins from ATC-amino acids is controlled by H_3O^+ concentration. Depending on pH, differences in reaction rates of the additions are determined by either $pK_a(NH_2)$ of amino compounds or electrical effects and steric hindrance of the molecules. The latter factors are crucial also for differences in cleavage rates of ATC-peptides. With regard to the pK_a values and simultaneous AITC decomposition by aqueous nucleophiles, the reactions with amino acids and oligopeptides are predominant reaction pathways of AITC in solutions of pH 10 and 8, respectively. Reaction mechanism of the cleavage of 2-thiohydantoins from ATC-peptides in alkaline and mild acidic solutions is different from the conventional Edman scheme used for anhydrous acid medium.

Keywords: *Allyl isothiocyanate; alanine; glycine; oligopeptides; N-allylthiocarbamoyl amino acids; N-allylthiocarbamoyl peptides; 2-thiohydantoins; allylthiourea; HPLC; Edman degradation*

INTRODUCTION

Allyl isothiocyanate (AITC) is formed from glucosinolate sinigrin naturally occurring in plants of the Brassicaceae family. It is a main pungent compound of some relishes, such as horseradish products and mustard pastes produced from black and brown mustard seeds (Velíšek, 1995). AITC is a strong electrophilic reagent and reacts easily with nucleophiles such as amines, amino acids, alcohols, water, and sulfites during food treatment and under physiological conditions (Drobnica et al., 1977; Cejpek et al., 1998).

In aqueous media, AITC gradually decomposes to unpungent or malodorous products (Pecháček et al., 1997; Chen and Ho, 1998). Investigation of the reactions of AITC with proteins, peptides, and amino acids is important with regard to nutritional, organoleptic, as well as technological changes of foods. For example, the presence of AITC in defatted meal of rapeseed is undesirable due to formation of indigestible and/or toxic products and with regard to a decrease of protein utilization of the fodder (Heaney and Fenwick, 1987). Similar compounds can be formed during food processing as well as in the gastrointestinal tract.

Interactions of AITC with thiol and disulfide groups of some enzymes and other biologically active peptides have been primarily studied (Kawakishi and Kaneko, 1987; Kroll et al., 1994). However, reactions with free amino groups imply formation of N,N′-disubstituted thiourea (*N*-thiocarbamoyl derivatives of peptides or amino acids) and several other secondary products which can affect nutrition as well (Drobnica and Augustin, 1965).

AITC reacts with amino acids and peptides to give *N*-allylthiocarbamoyl amino acid (ATC-amino acid) and ATC-peptide, respectively. The amino compounds take part in the reaction (Figure 1) only as free bases, the concentration of which is controlled by the dissociation reaction 1, which precedes the addition (2). The latter is followed by reaction 3, yielding a derivative of 3-allyl-2-thiohydantoin and, when a peptide reacts, the intact peptide chain minus the original NH_2-terminal residue as secondary products.

Reactions of isothiocyanates with amino acids are frequently used in analytical practice. Some isothiocyanates have been used in protein chemistry as reagents for the determination of primary structure of peptides and proteins (Edman, 1956) and in amino acids analysis via derivatization with phenyl isothiocyanate (e.g., Cohen and Strydom, 1988).

This work has focused on reactions of AITC with alanine, glycine and several di- and tripeptides, primary structures of which consist of glycine (G) and/or alanine (A), viz. glycylglycine (GG), glycylalanine (GA), alanylglycine (AG), glycylglycylglycine (GGG) and glycylglycylalanine (GGA), respectively, in aqueous model systems. The oligopeptides tested were selected to investigate formation of the adducts and the cleavage (secondary) products and confirm supposed reaction pathways. The reaction rates at various pHs were evaluated and the influence of amino acid structure and their sequence in the peptides on rates of both elementary reactions were documented.

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10.1021/jf991019s CCC: $19.00
Published on Web 07/08/2000

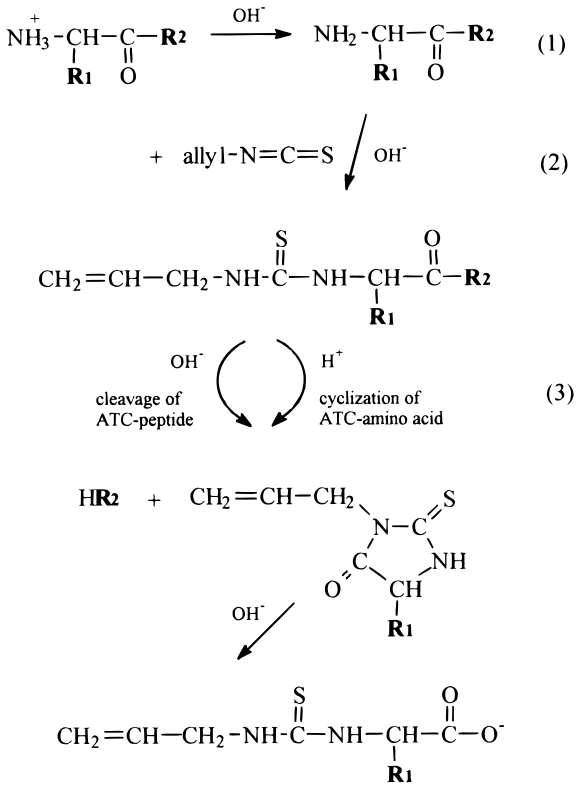

Figure 1. Reaction of AITC with peptides and amino acids.

EXPERIMENTAL PROCEDURES

Model Experiments. Solutions with equimolar concentrations of both reactants, i.e., 5 mmol/L AITC and 5 mmol/L of an amino compound in buffered aqueous solutions of pH 6.0 (citric acid/$Na_2HPO_4 \cdot 12H_2O$), 8.0 ($KH_2PO_4/Na_2B_4O_7$), and 10.0 ($Na_2CO_3/Na_2B_4O_7$), were prepared and stored at 25 ± 1 °C in closed bottles for several weeks. Allyl isothiocyanate (Fluka) was declared as >98% (m/m), but actual AITC stock purity was 84% because the concentrated AITC undergoes isomeration to allyl thiocyanate. The latter is not involved in reactions with amino compounds under the conditions used. In aqueous reaction systems, the isomeration further proceeds and contributes to overall AITC decrease (Pecháček et al., 1997).

Synthesis of ATC-amino Acids (ATC-peptides) and Corresponding 2-Thiohydantoins. 2-Thiohydantoins were synthesized according to the procedure of Mizrach and Polonska (1987) using allyl isothiocyanate and an amino compound. The product was purified by a repeated crystallization from hexane/toluene (1:1, v/v). Isolation of ATC-amino compounds synthesized according to the procedure of Kumar et al. (1988) was not successful due to considerable unstability of the products during water removal. Therefore ATC-amino acids were prepared by reaction of AITC with glycine at 80 °C in buffer of pH 10 and isolated after 10 min of reaction by semipreparative chromatography (with Lichrospher 100 C_{18} in LichroCart 250-10, E. Merck) from MeOH/water. Structure and purity of the synthesized compounds were confirmed by ^1H and ^{13}C NMR spectrometry and GC/MS analysis.

HPLC/UV Analysis. All analytes except parent amino compounds and allylamine were analyzed simultaneously by RP-HPLC/UV with Nova-Pak C18, 250 × 4.6 mm, 4 μm, with Guard-Pak Nova-Pak C18 (Waters). Gradient elution was performed by mixing of 0.05 mol/L buffer ($NaOH/KH_2PO_4$) of pH 6.5 and acetonitrile at a flowrate of 0.7 mL min^{-1}, ambient temperature, 20 μL injection and λ = 240 nm using HPLC system (Thermo Separation Products) consisting of binary system of high-pressure pumps constaMetric 3500 and constaMetric 3200 equipped with high-pressure gradient dynamic mixer (Watrex Praha, CZ), autosampler AS 100 and spectrophotometric detector spectroMonitor 3200. The reliability of the chromatographic separation was confirmed by peak purity and UV spectra comparisons of the analytes in a Waters 2 × 515 HPLC pump and 996 PDA detector system with the same column and eluent. Molar extinction coefficient of ATU was used for calculation of concentration of any ATC-amino compounds assuming very close ϵ values for them in the region of 240 nm (Edman, 1970).

GC/MS Analysis. To confirm structure of the analytes, GC/MS analyses were carried out on GCD system G1800A (Hewlett-Packard) with HP-5 (25 m, 0.25 mm, 0.25 μm), helium flow 0.6 mL/min, t_{inj} = 220 °C (120 °C for samples containing allylthiourea and diallylthiourea, when allylamine should be detected), t_{det} = 250 °C, column temperature program 40 °C, raised at 5 °C/min to 140 °C, then raised at 10 °C/min to 240 °C and held. 2-Thiohydantoins were determined directly, ATC-amino acids as bis(trifluoroacetate)-2-thiohydantoins or *N*-acetyl-ATC-amino acid propylesters. The confirmation of allylthiourea and allylamine formation was carried out after trifluoroacetylation.

Propylation and Acetylation. One milliliter of acetyl chloride in 1-propanol (1:5, v/v) was added to the dried sample. The mixture was heated at 110 °C for 20 min, and the solvent was evaporated by nitrogen stream, repeatedly dissolved in 1-propanol and dried. Volumes of 0.5 mL acetone, 0.2 mL triethylamine and 0.1 mL acetic anhydride were added to the sample and acetyl derivatives were formed during heating at 60 °C for 5 min. After solvent evaporation, the mixture was dissolved in ethyl acetate.

Derivatization with Trifluoroacetic Anhydride (TFAA). After solvent evaporation, 0.3 mL of acylation reagent (TFAA/dichloromethane 1:3, v/v) was added and left at laboratory temperature for 30 min.

RESULTS AND DISCUSSION

Depending on pH and concentrations of the reactants, reactions with amino acids and oligopeptides can be either the major or minor reaction pathway of AITC in aqueous solutions. The most important nucleophilic species competing with amino compounds in the model systems investigated are water and hydroxide ion. Kinetic measurements revealed that the AITC/amino compound reaction rates increase proportionally to growing OH^- concentration in a broad pH range (Table 1), while the rate of addition of the aqueous species shows a stagnation within pH 6−8 (see also Cejpek et al., 1997; Pecháček et al., 1997). Thus AITC decomposition made by aqueous nucleophiles is dominant in solutions of pH 6 − only up to 8% of AITC reacted with oligopeptides and/or amino acids. At pH 8, about two-thirds of AITC were retained in products originated from the reactions with amino compounds the basic forms of which are present in tenths of percent (i.e., tri- and dipeptides). Only 40−50% of AITC were bound in the reaction products of oligopeptides in solutions of pH 10, while more reactive amino acids reacted still with two-thirds of AITC under conditions used.

Time courses of both principal products arising from the reaction of AITC with glycine (allylthiocarbamoyl glycine, ATCG, and 3-allyl-2-thiohydantoin, ATH, in graphs *a* and *c*, respectively) and alanine (allylthiocarbamoyl alanine, ATCA, and 3-allyl-5-methyl-2-thiohydantoin, MeATH, in graphs *b* and *d*, respectively) are shown in Figure 2. As regards to the AITC/glycine system at pH 10, the concentration of ATCG reached a maximum (more than 60% yield) after 2 days and then

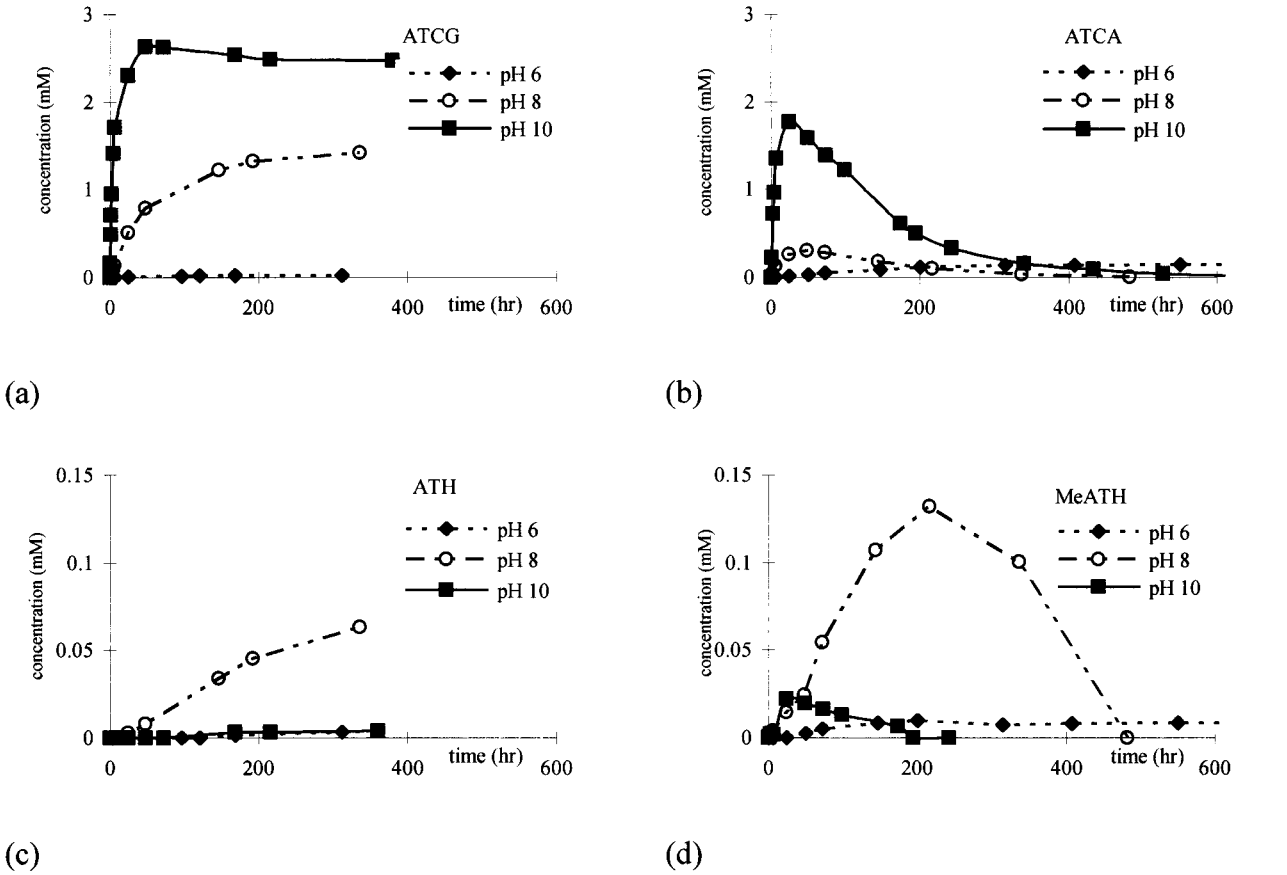

Figure 2. Formation and disappearance of principal products of the reaction of AITC with glycine (a, c) and alanine (b, d) in model solutions of different pH (25 °C).

Table 1. Apparent Reaction Rate Coefficients for (a) Overall AITC Decrease Caused by a Nucleophile (k_1') and (b) a Primary Reaction between AITC and Amino Compound (k_2')

		(a) $k_1' \times 10^5$ (s^{-1})			(b) $k_2' \times 10^3$ ($M^{-1}\,s^{-1}$)		
nucleophile	pK_a value[a]	pH 6	pH 8	pH 10	pH 6	pH 8	pH 10
H_2O, OH^-		0.30 ± 0.02	0.21 ± 0.01	1.64 ± 0.08			
A	9.69	0.17 ± 0.03	0.7 ± 0.1	6.4 ± 0.5	0.0050 ± 0.0005	0.381 ± 0.003	6.1 ± 0.3
G	9.6	0.05 ± 0.03	0.13 ± 0.03	4.5 ± 0.5	0.0034 ± 0.0001	0.508 ± 0.005	13.1 ± 0.5
AG	8.24	0.15 ± 0.02	0.6 ± 0.2	2.5 ± 0.5	0.0253 ± 0.0002	1.41 ± 0.01	2.0 ± 0.1
GA	8.2	0.10 ± 0.08	0.57 ± 0.08	2.0 ± 0.5	0.0517 ± 0.0003	3.9 ± 0.1	4.4 ± 0.1
GG	8.17	0.14 ± 0.08	0.6 ± 0.1	2.0 ± 0.2	0.069 ± 0.003	3.6 ± 0.3	8.6 ± 0.6
GGA	8.04	0.09 ± 0.02	1.3 ± 0.2	1.3 ± 0.2	0.070 ± 0.003	4.1 ± 0.1	5.2 ± 0.2
GGG	7.91	0.41 ± 0.02	2.1 ± 0.2	8.6 ± 0.9	0.203 ± 0.001	6.4 ± 0.3	10.0 ± 0.3

[a] *The Merck Index* (1996).

slowly decreased. Very low levels (<0.2% (molar) of AITC derived compounds) of ATH were found after two weeks of storage in solution of pH 10. At pH 8, about one-third of AITC reacted with glycine within two weeks and ATH concentration reached 1.3% in the same time. Less than 1% of AITC was incorporated into ATH and/or ATCG at pH 6.

In AITC/alanine systems of pH 8 and 10, a complete transformation of MeATH and/or ATCA to other products occurred within 3 and 4 weeks, respectively (Figure 2b,d). The products of the conversion are at least two unidentified compounds, which are quite stable in mild alkaline media and possess MeATH-like character when analyzed with HPLC/diode array system. Moreover, though alanine reacts with AITC slower than glycine at pH 8 and 10, the total elimination of AITC in alanine containing solutions is more rapid than in the presence of glycine (Table 1). At least in AITC/alanine systems, it implies another reaction of AITC with amino acid or its transformation product.

Though the main reason for the disappearance of ATC-amino acids and 2-thiohydantoins from solution is their mutual conversion (Cejpek et al., 1997) and, in alanine or alanyl containing systems, the transformation described above, several other decomposition pathways of the principal products were observed. One of the byproducts identified, allylamine (AA), is originated during alkali hydrolysis of 2-thiohydantoins together with the original amino acid and carbonyl sulfide. Besides it, AA is also a common product of the decomposition of AITC by aqueous nucleophiles (Kawakishi and Namiki, 1969).

Allylthiourea (ATU) was identified as another significant byproduct both in any reaction mixture and in a solution of pure 2-thiohydantoin and ATC-amino acid. For example, ATU, stable in alkaline media, comprised up to 30% of original AITC after 4 weeks in solution of pH 10 with alanine. ATU can be formed in higher amounts in alkaline media probably by reaction of AITC with ammonia arising from the Strecker degradation

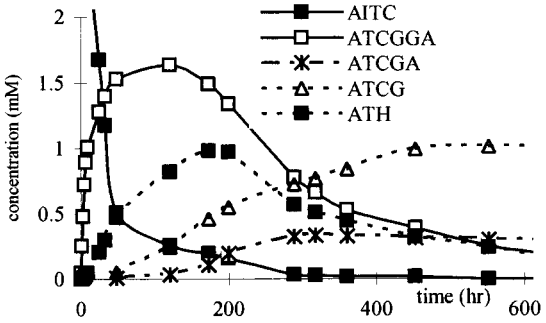

Figure 3. Changes in composition of reaction mixture of AITC and GGA during storage (pH 8, 25 °C).

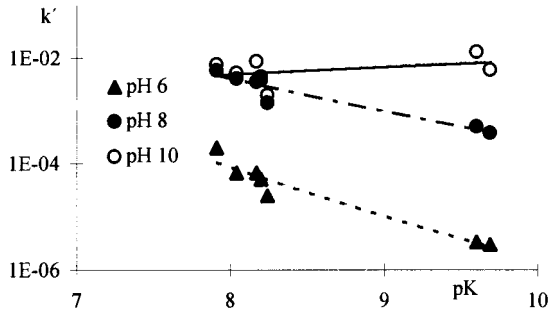

Figure 4. Effect of $pK_a(NH_2)$ values of amino acids and peptides on their reactivity toward AITC (expressed as k_2' in $M^{-1}\ s^{-1}$) in model solutions of different pH (25 °C). Details see in Table 1.

of an amino acid, though another mechanism has been also postulated (Ware, 1950). Formation of oxo-analogues of the main products by means of desulfuration was not confirmed at laboratory temperature.

Reactions of AITC with oligopeptides were carried out similarly to the reactions with amino acids. In addition to a primary adduct (ATC-peptide), products of the cleavage derived from NH_2-terminal amino acid, viz. 2-thiohydantoin and its open form − ATC-amino acid, appeared in the reaction mixture. Spectrum of the primary and secondary products may be extended by analogous compounds arising from progressive AITC degradation of a peptide chain until AITC is available (Figure 3). In solutions of pH 6, the reaction with a peptide chain is a minor reaction pathway for AITC since only 3% (in AG containing solution) to 8% of AITC (in GGG system) take part in the reaction. 2-Thiohydantoins were found in low concentrations corresponding to 0.1% (GA) to 2.4% (GGG) of AITC involved and they were considerably stable. In pH 8, 2-thiohydantoins incorporated 4−28% of AITC in their maxima and then gradually converted to their open forms (Figure 3). Negligible concentrations of 2-thiohydantoins (less than 1% yield) were detected in an oligopeptide containing solutions of pH 10 due to fast transformation to the corresponding ATC-amino acids. After some time, when cleavage reactions are finished, ATC-amino acids are the only primary and/or secondary products detected in alkaline solutions. For example, only ATCG occurred in GGG (having kept 39% of AITC), GG (34%), and GGA (34%) systems of pH 10 after 10, 21, and 41 days, respectively.

The degradation rate of AITC alone in buffered aqueous solutions is controlled by pseudo-first-order reaction kinetics in sufficiently low AITC concentration range (Ohta et al., 1995), as our data have also confirmed. The overall calculated apparent rate coefficients k_1' (Table 1) involve AITC consumption caused by addition and other reactions comprising the amino compounds. Following kinetics data, various abilities of the amino acids and peptides to decrease the AITC concentration in aqueous solutions are evident. In pH 10, when a base form of each amino compound predominates, they eliminate AITC in descendent order: GGG > A > G > AG > GG ≅ GA > GGA.

The addition reactions of AITC with amino compounds appear to follow the second-order relationship suggested by the stoichiometric equation. However, the formation of primary products in aqueous solutions containing an amino acid or a peptide does not fit exactly the equation for the second-order reactions even in early data range. The kinetics data inconsistence may be caused by almost immediate consecutive reactions involving primary products. Another possible reason has been outlined by Arnold et al. (1957) who demonstrated that the reaction of isocyanates exhibits anomalous kinetics rather then the simple second-order reaction and that it may be catalyzed by the products with urea structure arising from the reaction. Therefore apparent second-order constants (k_2') for comparison of reaction rates of the formation of primary products were used.

As denoted in our previous paper (Cejpek et al., 1997), temperature is a crucial factor affecting the addition rate constants (k_2'). For example, reactions of glycine with AITC were by about 2 orders of magnitude faster at 80 °C than at 25 °C. As evident from the apparent rate coefficients comprised in Table 1, the reaction rates depend strongly also on pH of the medium and hence on the amounts of unprotonated forms of amino compounds that vary with pH. It is generally assumed that the basicities of α-amino groups are the main factor determining the reactivity of amino compounds. This relationship was investigated in several earlier studies involving reactions of various aromatic isothiocyanates with amino acids and peptides resulting in more or less sufficient correlation coefficients (Drobnica et al., 1977).

Due to their lower pK_a values, oligopeptides reacted with AITC more rapidly than amino acids at pH 8 and below, where differences between them in NH_2-/NH_3^+- ratio are 1−2 orders of magnitude. Good values for linear correlation coefficients at pH 6 and 8 (0.97 and 0.95, respectively) calculated from the relationship between pK_a values and logarithms of the apparent rate constants of the reactions (k_2') were obtained. Similar correlation (0.92, and 0.98 if AG results were excluded) was achieved in solutions of pH 8 when the data of several other amino acids were included into the relationship (Cejpek et al., 1999).

At pH 10, where concentrations of the true nucleophiles (base forms) of all amino compounds are comparable, alanine and glycine show higher reactivity according to their higher basicity (Figure 4). However, no actual linear dependence of log k_2' on pK_a values at this pH occurred ($r = 0.40$). Moreover, the order of decreasing reactivity of amino compounds investigated at pH 10 (GGG > G > GG > A > GGA > GA > AG, Table 1) does not correspond at all to the order based on the pK_a values (A > G > AG > GA > GG > GGA > GGG). It follows from it that the differences in reactivity of the amino compounds at this pH are determined by steric hindrance and electrical effects.

Although a cleaving-off of alkylated *N*-terminal amino acids in alkaline or neutral solutions has already been described, it is not expected to be significant for non-

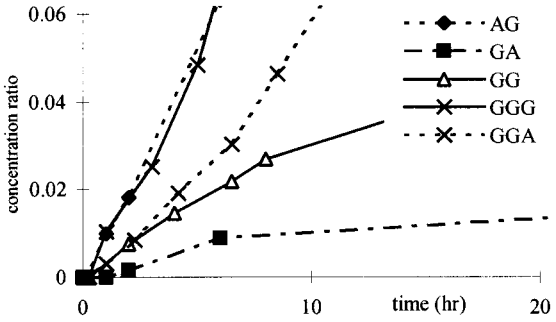

Figure 5. Comparison of cleavage rates of ATC-peptides. The rates are expressed as time-dependent ratio of summary concentration of 2-thiohydantoin and consecutive products to concentration of parent ATC-peptide (pH 8, 25 °C).

alkylated amino acids in connection with the Edman degradation (Törnqvist et al., 1986). However, our results document that the rates of cleavage of 2-thiohydantoins from ATC-peptides rise proportionally to pH within pH range 6−10. The rates in pH 8 and 10 are approximately by one and 2 orders of magnitude higher, respectively, than in pH 6 for each oligopeptide tested. The cleavage rates have been compared through the ratio of the sum of any secondary products (2-thiohydantoins, ATC-amino acids and their consecutive products) to primary product observed in early reaction solutions. The tendency to split off the NH_2-terminal amino acid in the form of 2-thiohydantoin at pH 8 (and also at pH 6) decreases in the order AG ≅ GGG > GGA ≅ GG > GA (Figure 5). At pH 10, ATCGGG is decomposed more rapidly than the other ATC-peptides, which evokes its enhanced sensitivity toward hydrolysis catalyzed by OH^-. The order of the decrease of cleavage rate is then changed to GGG ≫ AG > GG > GGA > GA.

Inductive and field effects of side-chain substituents of a peptide (amino acid) are expected to make differences in the rates of cleavage of the primary products. The intrinsic factors affecting the cleavage rate are partly the character of the amino acid side-chain on the α-carbon of NH_2-terminal amino acids and partly the structure of the rest of peptide chain. It is well demonstrated when comparing data from the systems of the dipeptides investigated. Though ATCGA formation is faster than that of ATCAG, MeATH appears earlier than the ATH analogue. In comparison to NH_2-terminal glycine, the presence of the methyl group of NH_2-terminal alanine accelerates the scission whereas alanine adjacent to the NH_2-terminal amino acid decreases the reaction rate (comp. GA vs GG data in Figure 5). The structure of the NH_2-terminal amino acid possesses a more significant effect on cleavage rate than the character of the rest of a peptide. The effect of amino acid alkyl residues diminishes with increasing distance from the reaction center (e.g., compare GA vs GGA data).

The acquired data have also revealed that the reaction mechanism of the cleavage of 2-thiohydantoin derivative arising from glycine or alanine in neutral and alkaline environment does not obey the reaction sequences suggested by Edman (1956). The reaction scheme introduced by Edman involves the formation of ATC-amino acid as an intermediate originated from unstable 2-thiazolinone in acidic medium. The former relatively slowly passes to the 2-thiohydantoin derivative. Under conditions used in our experiments no ATC-amino acid before the corresponding 2-thiohydantoin was formed (e.g., Figure 3).

CONCLUSIONS

In studying reactions of AITC with α-NH_2 groups of amino acids and peptides, it has been demonstrated that not only the AITC addition but also the 2-thiohydantoin cleavage rates rise proportionally to pH within pH range 6−10. The products correspond to those arising from the conventional Edman peptide degradation method or its modifications using acidic media for cyclization/cleavage and conversion. Another difference in comparison to the Edman scheme has been found: ATC-amino acid does not occur as an intermediate during 2-thiohydantoin formation via 2-thiazolinone in neutral and alkaline aqueous solutions as supposed in acidic media.

Besides the principal products (i.e., ATC-peptides, 2-thiohydantoins, and ATC-amino acids), some other compounds arising from the reactions of the amino compounds with AITC, viz. allylamine (AA) and allylthiourea (ATU), were identified. Moreover, 2-thiohydantoins arising from some peptides and amino acids (e.g., alanine) can be easily converted to still unidentified products in neutral and alkaline media. Further effort will be focused on investigation of these consecutive reactions.

The presented data provide useful information to extend knowledge on stability and mutual conversion of both open and cyclic forms of ATC-amino acids in mild acidic to alkaline pH range. It will be useful for evaluation of reaction yields and adjusting of pH and duration of the addition (coupling) reaction when methods of amino acids determination and/or Edman degradation are applied.

Although the formation of ATC-peptides and particularly ATC-amino acids is favored under alkaline conditions, the adducts and the cleavage products have been also found in neutral and mild acidic media in more or less significant levels depending on $pK_a(NH_2)$ of parent amino compounds. Particularly in food systems containing limited amounts of compounds with thiol and disulfidic groups, the reactions of NH_2 groups with AITC can cause significant changes in nutritional value of proteins, their digestibility and AITC-related organoleptic profile during food treatment and under physiological conditions.

ABBREVIATIONS USED

A, alanine; AA, allylamine; AG, alanylglycine; AITC, allyl isothiocyanate; ATCA, allylthiocarbamoyl alanine; ATC-amino acid, *N*-allylthiocarbamoyl amino acid; ATC-peptide, *N*-allylthiocarbamoyl peptide; ATCG, allylthiocarbamoyl glycine; ATCGA, allylthiocarbamoyl glycylalanine; ATCGG, allylthiocarbamoyl diglycine; ATCGGA, allylthiocarbamoyl glycylglycylalanine, ATCGGG, allylthiocarbamoyl triglycine; ATH, 5-allyl-2-thiohydantoin; ATU, allylthiourea; G, glycine; GA, glycylalanine; GG, glycylglycine; GGA, glycylglycylalanine; GGG, glycylglycylglycine; ITCs, isothiocyanates; MeATH, 5-allyl-3-methyl-2-thiohydantoin; TFAA, trifluoroacetic anhydride.

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Received for review September 15, 1999. Revised manuscript received April 26, 2000. Accepted May 13, 2000. This work was supported in part from research grant no. 525/96/0163 provided by the Grant Agency of Czech Republic.

JF991019S