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N-Bromosuccinimide assisted oxidation of tripeptides and their amino acid analogs: Synthesis, kinetics, and product studies

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ABSTRACT

Three tripeptides (TPs), Gly–Val–Gly, Ala–Val–Gly and Gyl–Phe–Gly, were synthesized and characterized. The kinetics of oxidation of the TPs and their constituent amino acids (AAs) by *N*-bromosuccinimide (NBS) was studied in the presence of perchloric acidic medium at 28 °C by following the reaction spectrophotometricaly at λ_{max} = 240 nm. In all cases, the kinetics of reactions was first-order with respect to each [NBS] and [AA] or [TP]. Increased [H^+], succinimide (the reduction product of NBC), chloride and perchlorate concentrations had no effect on the rate of reactions. However, the reaction rate was increased with increased dielectric constant of the medium and with the hydrophobicity of AAs and TPs. Activation parameters for each of the substrate were considerably different. Analysis of the oxidation products indicated that the amino acids and peptides underwent oxidative deamination and decarboxylation to form corresponding aldehydes. Based on these data, plausible mechanisms for the oxidation AAs and TPs are proposed.

1. Introduction

Oxidative reactions play an important role in a variety of biochemical events ranging from normal metabolism to ageing and disease process [1,2]. Peptides and proteins represent major targets for modification in these reactions and the identification of sites and structures of modifications may lead to a mechanistic understanding and approaches for prevention. In this context, oxidation of α-amino acids is one of the well-documented biochemical processes. Several studies have been reported on the kinetics of oxidation of various substrates by *N*-bromosuccinimide (NBS) in different media [3–13]. Extensive work has been reported on the kinetics of oxidation of amino acids, peptide sequence with various metal ions and other oxidants [14–16].

Previously, we reported the comparative studies on the kinetics of oxidation of amino acids and dipeptide [17]. Quite recently we also reported the comparative studies on the kinetics of oxidation of amino acids and tetrapeptides by NBS [18]. However, comparative studies on the kinetics of oxidation of amino acids and tripeptides by NBS have not been reported. Therefore, we extended our investigation on the kinetics of oxidation of tripeptide and their constituent amino acids by NBS. In the present studies, the oxidative behavior of NBS towards tripeptides in perchloric acid medium has been studied extensively. Besides, we made comparative studies on the kinetics of oxidation of tripeptide and their constituent amino acids.

The cross-linked polytripeptide matrices based on the repeating amino acid sequences, Gly–Val–Gly, Ala–Val–Gly and Gyl–Phe–Gly were tested for cell adhesion promoting activity in both the absence and presence of fetal bovine serum [19]. The degree of cell attachments increases with the increase in hydrophobicity. In this context, it was thought to be interesting to investigate the hydrophobicity dependent oxidative behavior of NBS towards these tripeptides and their constituent amino acids.

2. Experimental

Peptides were synthesized by classical solution phase methods [20]. The *tert*-butyl-oxycarbonyl (Boc) group was used for temporary N^{α}-protection and its removal was achieved with 4 M HCl in dioxane or trifluoroacetic acid. The C-terminal carboxyl group was protected by the benzyl ester and its removal was effected by hydrogenolysis using $HCOONH_4$-Pd/C (10%) [21]. All coupling reactions were achieved with isobutyl chloroformate. The protected peptides were purified by the crystallization and characterized by physical and analytical techniques [20]. The purity of the free peptides was checked by paper chromatography and HPLC.

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All the amino acids used except glycine were of L-configuration unless specified otherwise. All *tert*-butyl-oxycarbonyl (Boc) amino acids, amino acid derivatives and trifluoroacetic acid (TFA) were purchased from Advanced Chem. Tech., (Louisville, KY, USA). Isobutyl chloroformate and *N*-methylmorpholine (NMM) were purchased from Sigma Chemicals (St. Louis, MO, USA). All solvents and reagents were of analytical grade or were purified according to the procedure recommended for peptide synthesis. Thin-layer chromatography (TLC) was carried out on silica gel plates obtained from Whatman, with the following solvent systems: chloroform–methanol–acetic acid (90:10:3); R_f^1 and chloroform–methanol–acetic acid (85:15:3); R_f^2. The compounds on TLC plates were detected by UV light, spraying with ninhydrin or by chlorine–toludine. Paper chromatography was carried out on Whatman No. 1 chromatographic paper with the solvent system butanol–acetic acid–water (4:1:5, upper phase). The compounds on paper were detected by spraying with ninhydrin. Melting points (uncorrected) were determined with a Selaco Can. No. 103 melting-point apparatus. Elemental analyses were carried out by Mic Anal (Tucson, AZ, USA). Optical rotations were measured using a PerkinElmer Model 243 digital polarimeter. Amino acid analysis of the sample was performed on a Waters HPLC Pico-Tag analyzer using 6.0 M HCl containing 1% (v/v) phenol at 110 °C for 72 h in a sealed tube under vacuum from which the air had been removed using nitrogen. For each reaction the product analysis was carried out by gas chromatography (GC 15A, Shimadzu, Kyoto, Japan). All tripeptides were synthesized according to the procedure published by Andru and Merrifield [20].

2.1. Preparation of NBS solution

An aqueous solution of NBS was prepared afresh each day from a GRS Merck sample and its strength was checked by the iodometric method [22]. Aqueous solutions of AAs and TPs of known concentrations were prepared. Known strength of aqueous solution of mercuric acetate and succinimide was also prepared. All other reagents were of analytical grade. Double distilled water was used throughout the investigation.

2.2. Kinetic measurements

The kinetic studies were carried out in glass-stoppered pyrex boiling tubes under pseudo-first-order conditions with [AAs] or [TPs] ≫ [NBS]. The reactions were initiated by the rapid addition of known amounts of oxidant solution, pre-equilibrated at a desired temperature, to mixtures containing the required amount of AAs or TPs, perchloric acid, succinimide and water in the boiling tube, thermostated at the same temperature. The progress of the reaction was monitored for at least two half-lives by measuring the absorbance of unreacted oxidant at 240 nm using a spectrochem Elico SL 150 UV–Vis spectrophotometer. The reaction mixture was quenched appropriately [23]. The pseudo-first-order rate constant (k_{obs}) was calculated by graphical methods and the values were reproducible to within ±3% error. Same procedure was followed for blank using water as blank.

3. Results

3.1. Dependence of the rate on [NBS], [AA] and [TP]

All kinetic runs were performed under pseudo-first-order conditions with $[AA]_o \gg [NBS]_o$ and $[TP]_o \gg [NBS]_o$. Plots of log [NBS] versus time, which were linear with slopes 1.05, 0.99 and 1.08 for over 75% of the reaction, showing a first-order dependence of the rate on [NBS] (Table 1). At constant $[NBS]_o$, $[succinimide]_o$, $[HClO_4]_o$ and temperature, the rate increased with increase in $[AA]_o$ or $[TP]_o$ (Table 1). Plots of log k_{obs} versus log $[AA]_o$ (Fig. 1) were linear with slopes 1.00 [17], 0.99 [17], 1.01[18] and 0.98 [18] for Gly, Val, Ala and Phe, respectively. Plots of log k_{obs} versus log $[TP]_o$ (Fig. 1) were linear with slopes 1.11, 1.09, and 0.99 for Gly–Val–Gly, Ala–Val–Gly and Gyl–Phe–Gly, respectively.

Table 1
Effect of varying reactant concentration on the reaction rate with $[HClO_4] = 0.01$ mol dm^{-3}; [succinimide] = 0.1 mol dm^{-3}; $[Hg(CH_3COO)_2] = 0.001$ mol dm^{-3}; $T = 301$ K.

$[NBS] \times 10^6$ (mol dm^{-3})	$[S] \times 10^4$ (mol dm^{-3})	$k_{obs} \times 10^5$ (s^{-1})						
		Gly[b]	Ala[b]	Val[a]	Phe[b]	GVG	AVG	GFG
0.6	1.0	10.81	11.64	12.40	13.68	8.79	9.80	11.11
0.8	1.0	10.66	11.62	12.37	13.72	8.78	9.84	11.08
1.0	1.0	10.61	11.61	12.79	13.75	8.76	9.81	11.12
1.2	1.0	10.24	11.58	12.89	13.69	8.70	9.83	11.10
1.4	1.0	10.10	11.48	12.85	13.68	8.72	9.80	11.14
0.6	0.6	7.73	8.05	8.24	9.81	5.25	5.91	6.72
0.6	0.8	8.18	9.23	10.33	11.90	7.10	7.73	8.96
0.6	1.0	10.61	11.64	12.79	13.70	8.90	9.77	11.09
0.6	1.2	12.07	13.72	15.86	15.62	10.48	11.82	13.54
0.6	1.4	14.20	16.39	17.25	18.16	12.31	13.65	15.86
0.6	1.6	16.24	17.85	19.15	20.06	14.62	15.71	17.59

[a] Data taken from ref. [17].
[b] Data taken from ref. [18].

Table 2
Effect of varying dielectric constant on the reaction rate, with $[NBS] = 1.0 \times 10^6$ mol dm^{-3}; $[S] = 1.0 \times 10^4$ mol dm^{-3}; $[Hg(CH_3COO)_2] = 0.001$ mol dm^{-3};[succinimide] = 0.1 mol dm^{-3}; $[HClO_4] = 0.01$ mol dm^{-3}; $T = 301$ K.

MeOH (%, v/v)	Dielectric constant (D)	$k_{obs} \times 10^5$ (s^{-1})						
		Gly[b]	Ala[b]	Val[a]	Phe[b]	GVG	AVG	GFG
0	76.73	10.61	11.64	12.79	13.70	8.76	9.81	11.12
10	72.37	12.47	12.39	14.25	16.51	10.62	11.90	13.90
20	67.48	15.88	14.48	16.63	18.06	12.40	13.70	14.94
30	62.71	17.54	15.85	18.10	20.11	15.06	15.62	16.20
40	58.06	20.06	17.85	20.25	23.05	17.36	18.16	19.82

[a] Data taken from ref. [17].
[b] Data taken from ref. [18].

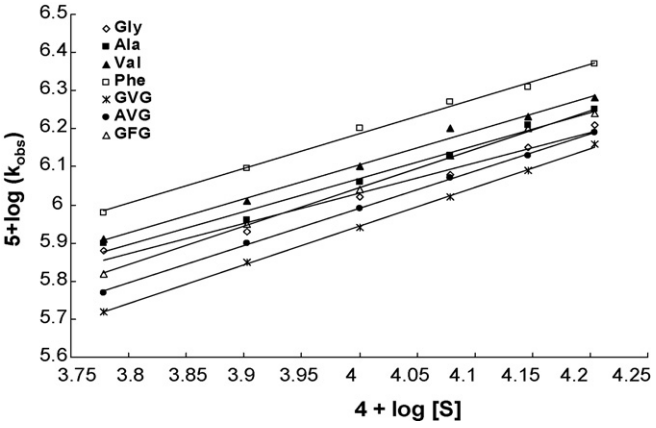


Fig. 1. Effect of [substrate] on the reaction rate.

3.2. Dependence of the rate on [acid]

Kinetic measurements were performed in $HClO_4$–$NaClO_4$ solutions of different $[H^+]$. The effective $[H^+]$ used was evaluated using the calibration curve of $[HClO_4]$ versus $[H^+]$. An increase in $[H^+]$ (from 0.01 to 1.0 M) had no effect on the rate.

3.3. Dependence of the rate on the reduction product and added salts

The effect on the rate of varying concentrations of succinimide (which is the reduction product of the oxidant, NBS) was investigated. An increase in [succinimide] (from 0.01 to 0.5 M) had no effect on the rate. This indicated that the product is not involved in a pre-equilibrium with the oxidant. Similarly, the effect of anions Cl^- (from 0.01 to 0.5 M) and ClO_4^- (from 0.01 to 1.0 M) on the rate was insignificant.

3.4. Effect of solvent composition

The dielectric constant (D) of the reaction medium was varied by changing the solvent composition with added methanol (0.0–40%). The rate increased with increase in methanol content (Table 2). The plots of $\log k_{obs}$ versus $1/D$ were linear ($r > 0.999$, $s \leq 0.01$) with positive slopes (Fig. 2). Measurements of rate constants for the oxidation by NBS were done both in the presence and absence of each substrate (AAs or TPs) and the rate constants were taken for the calculation of effective k_{obs}. The rate of oxidation of methanol in the absence of amino acids or TPs were negligible under the conditions used.

3.5. Activation parameters

To determine the activation parameters, the reactions were carried out at different temperatures (293–313 K, Table 3). Arrhenius plots of $\log k_{obs}$ versus $1/T$ (Fig. 3) which were linear with slopes 1.02, 1.05 and 0.99 used to calculate activation energies (E_a). Based on these values, the activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ along with the frequency factor ($\log A$) were evaluated (Table 4). The moderate values of $\Delta H^{\ddagger}$, a large negative $\Delta S^{\ddagger}$ and the firmly high $\Delta G^{\ddagger}$ support the mechanism. The near constancy of $\Delta G^{\ddagger}$ values indicates a solvated AAs or TPs and operation of a similar mechanism for the oxidation of all the AAs or TPs.

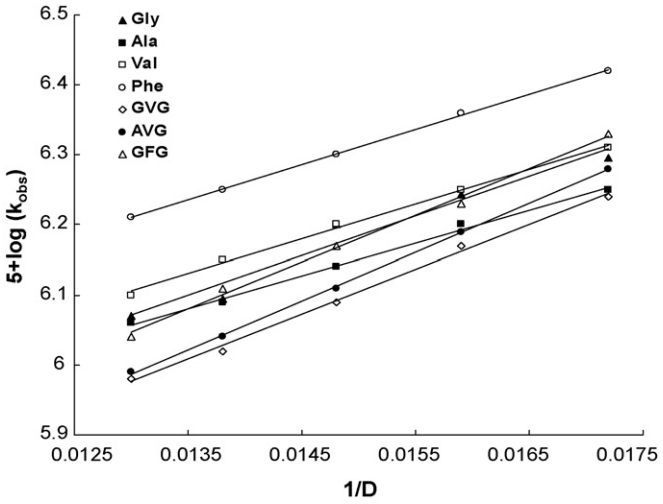


Fig. 2. Effect of dielectric constant (D) on the reaction rate.

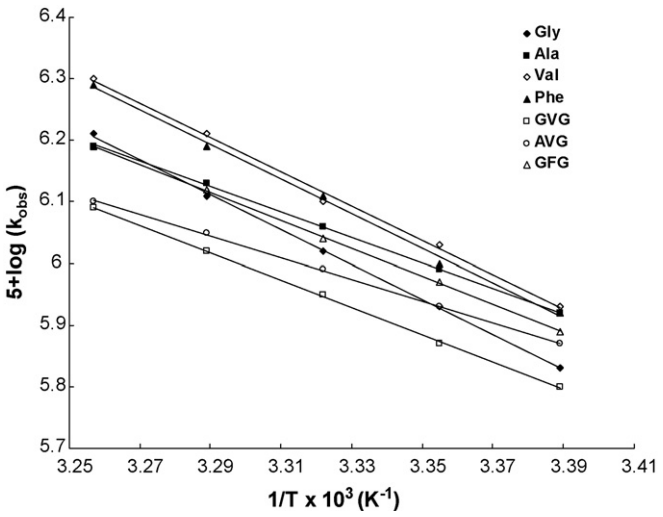


Fig. 3. Effect of temperature on the reaction rate.

Table 3
Temperature dependence of the oxidation of substrate with $[NBS] = 1.0 \times 10^6$ mol dm^{-3}; $[S] = 1.0 \times 10^4$ mol dm^{-3}; $[Hg(CH_3COO)_2] = 0.001$ mol dm^{-3}; [succinimide] = 0.1 mol dm^{-3}; $[HClO_4] = 0.01$ mol dm^{-3}.

Temperature (K)	$(1/T) \times 10^3$ (K^{-1})	$k_{obs} \times 10^5$ (s^{-1})						
		Gly[b]	Ala[b]	Val[a]	Phe[b]	GVG	AVG	GFG
295	3.389	5.89	8.46	8.41	8.68	6.34	7.12	7.84
298	3.355	7.86	10.48	10.23	10.81	7.50	8.51	9.42
301	3.322	10.61	11.39	12.79	13.70	8.79	9.81	11.11
304	3.289	13.54	14.46	15.67	16.19	10.18	11.24	13.18
307	3.257	16.47	16.23	18.10	20.44	12.28	12.75	15.71

[a] Data taken from ref. [17].
[b] Data taken from ref. [18].

Table 4
Activation parameters for the oxidation of substrate by NBS, with [NBS] = 1.0×10^{6} mol dm^{-3};[S] = 1.0×10^{4} mol dm^{-3};[Hg $(CH_3COO)_2$] = 0.001 mol dm^{-3}; [succinimide] = 0.1 mol dm^{-3}; [$HClO_4$] = 0.01 mol dm^{-3}.

Substrate	E_a (kJ mol^{-1})	$\Delta H^{\ddagger}$ (kJ mol^{-1})	$\Delta S^{\ddagger}$ (J mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (kJ mol^{-1})	log A
Gly[b]	70.25	67.75	−93.37	95.85	8.36
Ala[b]	64.78	62.27	−114.70	96.82	7.24
Val[a]	50.38	49.24	−122.85	94.90	6.40
Phe[b]	70.25	67.75	−93.40	95.87	8.35
GVG	34.83	32.33	−215.48	97.19	1.98
AVG	36.02	34.75	−207.58	97.23	2.39
GFG	38.77	36.27	−200.45	96.60	2.77

[a] Data taken from ref. [17].
[b] Data taken from ref. [18].

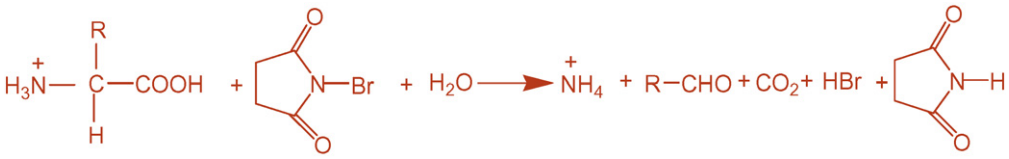
3.6. Test for free radicals

Addition of the reaction mixture to aqueous acrylamide monomer solutions did not initiate polymerization, indicating the absence of *in situ* formation of free radical species in the reaction sequence.

3.7. Reaction stoichiometry

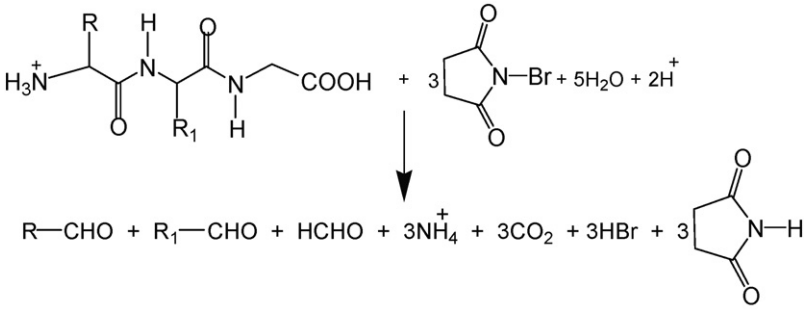
Reaction mixtures containing AA or TP (0.001 M), perchloric acid (0.1 M) and excess NBS (0.01 M) were kept for 24 h at 25 °C. The unconsumed NBS was then determined to calculate the stoichiometry ratios. One mole of oxidant was sufficient to oxidize 1 mol of AA and 3 mol of oxidant were sufficient to oxidize 1 mol of TP leading to products, aldehydes, carbon dioxide, ammonia and succinimide. Based on these results, the following stoichiometric equations (a) and (b) are suggested.

A general stoichiometric equation for four amino acids; (a) glycine (Gly); alanine (Ala); valine (Val); and phenylalanine (Phe).



where R = –H for glycine; R = $–CH_3$ for alanine; R = $–CH(CH_3)_2$ for valine and R = $–CH_2C_6H_5$ for phenylalanine.

A general stoichiometric equations for tripeptides: glycyl–valyl–glycine (Gly–Val–Gly), alanyl–valyl–glycine (Ala–Val–Gly) and glycyl–phenylalanyl–glycine (Gyl–Phe–Gly).



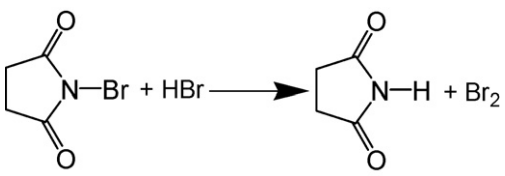
where R = H and R_1 = $–CH(CH_3)_2$ for GVG; R = –CH and R_1 = $–CH(CH_3)_2$ for AVG; R = H and R_1 = $–CH_2–C_6H_5$ for GFG.

3.8. Product analysis

After the reaction was completed, the reaction products were extracted with diethyl ether and subjected to column chromatography on silica gel (60–200 mesh) using gradient elution (dichloromethane to chloroform). R_f^1: chloroform–methanol–acetic acid (95:5:3); R_f^2: chloroform–methanol–acetic acid (90:10:3); R_f^3: chloroform–methanol–acetic acid (85:15:3). Aldehydes were determined qualitatively by gas chromatography. The retention values of formaldehyde, acetaldehyde, 2-methyl propanaldehyde, and phenyl acetaldehyde are 6.0, 5.14, 25.4 and 31.09, respectively, which are identical with those for authentic samples. Ammonia and CO_2 were detected by the conventional tests.

4. Discussion

The results of the oxidation of amino acids and tripeptides, recorded here, have revealed that the reactions have identical kinetics and thus appear to have common mechanism. Insignificant effect of mercuric acetate on reaction rate rules out its involvement in NBS oxidation and acts only as a scavenger [6,24] for any Br^- formed in the reaction. It suppresses completely the oxidation by Br_2, which would have been formed by the interaction of HBr and NBS as follow:

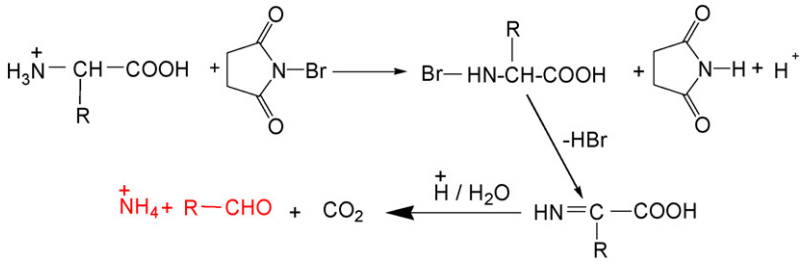


$$\text{NBS} + \text{AAs or TPs} \xrightarrow{k_1} \text{X} \quad \text{(i) slow and rds}$$

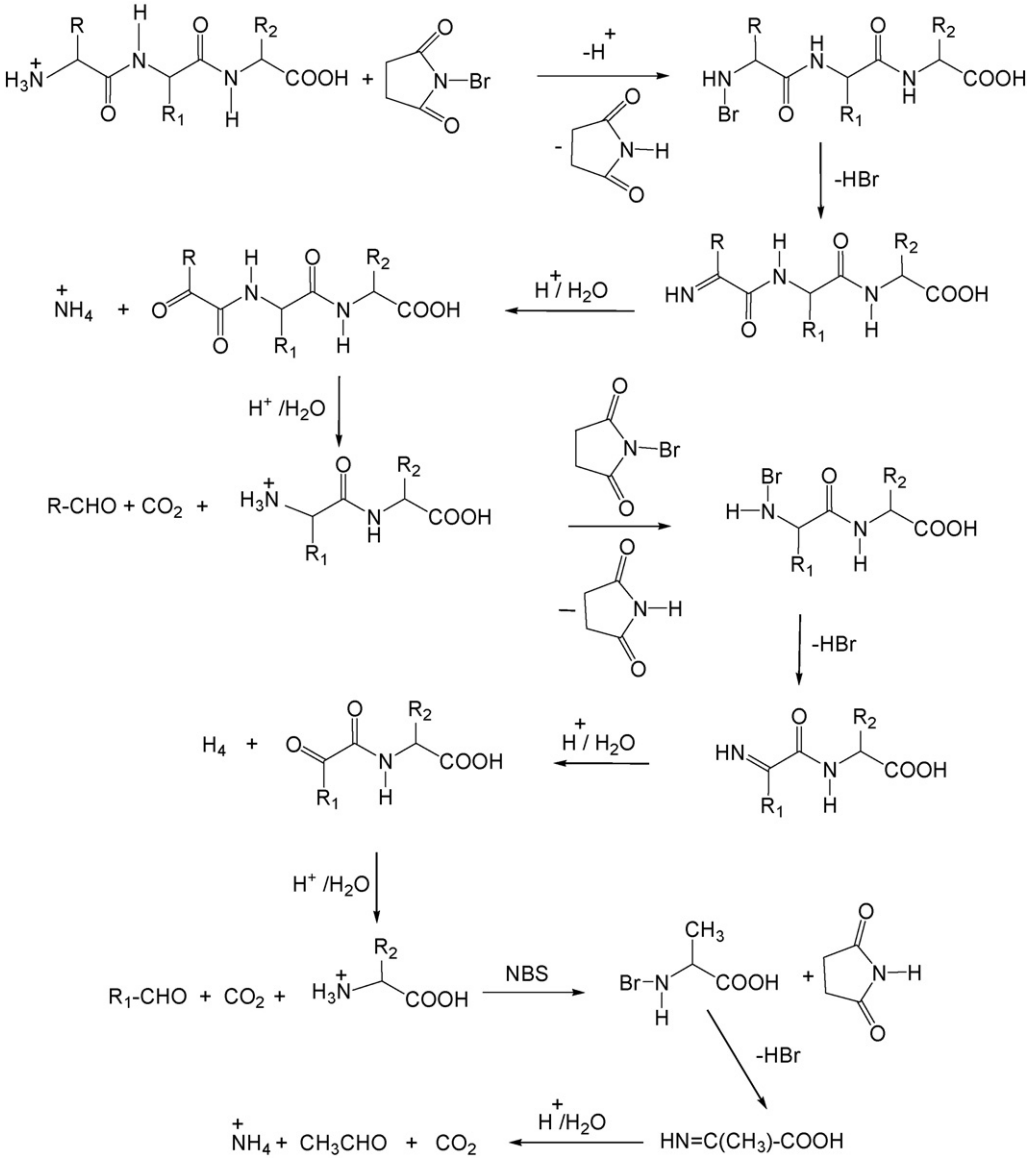
$$\text{X} \xrightarrow{k_2} \text{products} \quad \text{(ii) fast}$$

Hence, Rate = k_1 [NBS] [AAs or TPs]

Scheme 1. Rate = k_1 [NBS] [AAs or TPs].

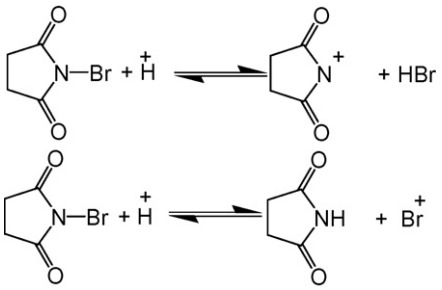


Scheme 2. R = –H for glycine; R = $-CH_3$ for alanine; R = $-CH(-CH_3)_2$ for valine; R = $-CH_2C_6H_5$ for phenylalanine.



Scheme 3. R = H and $R_1 = -CH(CH_3)_2$ for GVG; R = –CH and $R_1 = -CH(CH_3)_2$ for AVG; R = H and $R_1 = -CH_2-C_6H_5$ for GFG.

Mercuric acetate thus ensures the oxidation purely through NBS. NBS is known to exist in acidic media in the following equilibrium:



The possible oxidizing species of NBS in aqueous acidic solutions are: NBS itself, protonated NBS, (i.e., N^+BSH) and Br^+. In the presence of mercuric acetate protonated form of NBS, i.e. N^+BSH has been considered [25] as its reactive species in acidic medium.

In the present investigation, it was found that the added succinimide has a negligible effect on the rate of the reaction. This categorically excludes Br^+ as the oxidizing species, only if the reverse reaction is significant. Hence the active species may be NBS or N^+BSH. The order of the reaction with respect to $[H^+]$ is zero, and hence, N^+BSH does not participate in the rate-determining step. All these factors indicate that NBS is the only possible oxidant species taking part in the reaction. Scheme 1 accounts for the observed experimental results for AA and TP. In the light of the experimental results, a suitable mechanism for AA and TP in Schemes 2 and 3 has been proposed respectively.

5. Conclusion

The rates of oxidation of (AAs) and tripeptides (TPs) by NBS were compared under identical conditions. It was found that the rates of oxidation of tripeptide were slower than of free amino acids or monomers. The change is due to the increased distance between the functional groups and resultant weaker electrostatic effects. Furthermore, an apparent correlation was noted between the rate of oxidation and the hydrophobicity [26] of those peptide sequences where increased hydrophobicity results in an increased rate of oxidation. The probable reason for the increased oxidation rate for the more hydrophobic peptides is that the carboxylic groups are more destabilized enhancing the rate of formation of a transition state with NBS which in turn increases the oxidation rate.

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