α -Bromo-4-amino-3-nitroacetophenone, a New Reagent for Protein Modification

MODIFICATION OF THE METHIONINE-290 RESIDUE OF PORCINE PEPSIN

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A new coloured reagent for protein modification, α -bromo-4-amino-3-nitroacetophenone ($\text{NH}_2\text{BrNphAc}$), was synthesized. The reagent was found to alkylate specifically the methionine-290 residue of porcine pepsin below pH 3 at 37°C, which lead to a 45% decrease of enzyme's activity towards haemoglobin. The effect of this reagent as well as that of other phenacyl bromides on the activity of pepsin appeared to be a result of steric hindrance caused by the attachment of bulky reagent residue to the edge of the cleft harbouring the enzyme active site. Only marginal reaction with the ω -carboxy group of aspartic acid-315 was found under the above conditions. More pronounced esterification of carboxy groups (up to one residue per enzyme molecule) occurred when the pH was shifted to 5.2. The latter modification had no noticeable effect on enzyme activity, thus disproving a previously held assumption that pepsin inactivation by phenacyl bromide is due to the carboxy-group esterification. α -Bromo-4-amino-3-nitroacetophenone forms derivatives with characteristic u.v. spectra when it reacts with methionine, histidine, aspartic and glutamic acid residues, and may be recommended as a reagent for protein modification.

α -Bromoketones are occasionally used for the modification of nucleophilic groups in proteins (Glover *et al.*, 1976; Diopoli & Olomucki, 1972). Erlanger *et al.* (1965) studied the interaction of porcine pepsin with a series of α -haloketones. It had been found that the reaction of *p*-bromophenacyl bromide (the most thoroughly studied case) with the enzyme lead to incorporation of one inhibitor residue into protein molecule, with a concomitant 78% loss of enzyme activity. Since treatment with mercaptans efficiently re-activated the enzyme, it had been assumed initially that methionine or cysteine residues were involved in the reaction with phenacyl bromides. Later Gross & Morrel (1966) reported that *p*-bromophenacyl bromide esterified an aspartic acid residue and suggested the procedure for its localization in the enzyme primary structure. In a subsequent publication, Erlanger *et al.* (1966) clearly supported this explanation and gave the amino acid composition of a labelled peptide containing an aspartic acid residue presumably

labelled by *p*-bromophenacyl bromide (Gly₂, Asp, Ser, Glu₂). However, no fragment of corresponding composition was found in the amino acid sequence of pepsin (Sepulveda *et al.*, 1975a), thus making the identification of inhibitor-attachment site doubtful.

More recently published data on X-ray analysis of pepsin and pepsin-like proteinases from fungi (Andreeva *et al.*, 1978; Hsu *et al.*, 1977; Subramanian *et al.*, 1977; James *et al.*, 1977) stimulated interest in the chemical modification of these enzymes insofar as this approach might help to identify the functionally important groups and outline the topography of the active site.

The available data on the reaction of pepsin with phenacyl bromides being incomplete and contradictory, we decided to re-examine this reaction. To overcome the difficulties of reagent localization in the pepsin amino acid sequence and to facilitate the control of the reaction, we synthesized a new reagent, α -bromo-4-amino-3-nitroacetophenone ($\text{NH}_2\text{BrNphAc}$). Introduction of ortho-positioned amino and nitro groups into the phenacyl bromide structure endowed this reagent with capacity to form protein derivatives with characteristic u.v. spectra.

Abbreviation used: $\text{NH}_2\text{BrNphAc}$, α -bromo-4-amino-3-nitroacetophenone; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Experimental

Materials

Chromatographically pure porcine pepsin (Stepanov & Vaganova, 1969) and aspergillopepsin A (carboxylic proteinase from *Aspergillus awamori*) (Kovaleva *et al.*, 1977) were used. Thermolysin was purchased from Merck, Darmstadt, Germany, and *p*-aminoacetophenone from Chemapol, Prague, Czechoslovakia.

Methods

Proteolytic activity. This was measured at pH 1.8 with bovine haemoglobin as substrate (Anson, 1938).

T.l.c. This was carried out on Silufol plates with a toluene/acetic acid/water (2:1:1, by vol.) system.

***p*-Acetamidoacetophenone (I, Scheme 1).** A solution of 10 g of *p*-aminoacetophenone in 50 ml of acetic anhydride was stirred for 1 h at 20°C, then poured into 300 ml of cold water. The precipitate was collected and recrystallized from 20 ml of ethanol, yielding 11.5 g (90%) of compound (I). M.p. 171°C, reported 171°C (Bruining, 1922).

4-Acetamido-3-nitroacetophenone (II, Scheme 1). HNO₃ (40 ml) (*d* 20°C = 1.51) was added dropwise to acetic anhydride cooled to -15°C, then 8 g of *p*-acetamidoacetophenone were added in one portion. The mixture was stirred for 1 h at 0°C, then poured into 600 g of crushed ice. The yellow precipitate was filtered off, washed with cold water, dried over KOH and recrystallized from 100 ml of ethanol. Yield 6.5 g (65%), m.p. 139°C, *R_F* 0.20 (in toluene/acetic acid/water; see above). (Found: C, 53.91; H, 4.54; N, 12.62. C₁₀H₁₀N₂O₄ requires C, 54.05; H, 4.53; N, 12.60%.)

α -Bromo-4-acetamido-3-nitroacetophenone (III, Scheme 1). Bromine [1.1 ml (22.5 mmol)] in 2.2 ml of acetic acid was added dropwise with stirring to a solution of 5 g (22.5 mmol) of 4-acetamido-3-nitroacetophenone in 22 ml of acetic acid at 50–55°C. The mixture was then stirred for 1 h at room temperature, then poured into 200 ml of cold water. The precipitate was filtered off, dried over KOH and recrystallized from 35 ml of ethanol. Yield: 4.6 g (88%), m.p. 102°C, *R_F* 0.26 (Found: C, 40.28; H, 3.10; N, 9.27. C₁₀H₉N₂O₄Br requires C, 39.90; H, 3.01; N, 9.30%.)

α -Bromo-4-amino-3-nitroacetophenone (NH₂BrNphAc; IV, Scheme 1 and Table 1). A suspension of 3 g of α -bromo-4-acetamido-3-nitroacetophenone in 40 ml of 24% HBr was heated in a boiling-water bath for 5 min, then quickly cooled to 0°C. The precipitate was filtered off, washed with cold water, dried over KOH, then recrystallized from 35 ml of ethanol. Yield: 2.1 g (81%), m.p. 173°C, *R_F* 0.17. (Found: C, 37.21; H, 2.69; N, 10.47. C₈H₇N₂O₃Br requires C, 37.09; H, 2.72; N, 10.81%.)

α -Propionyloxy-4-amino-3-nitroacetophenone (V, Table 1). A solution of triethylamine (0.56 ml, 40 mmol) and propionic acid (0.48 ml, 42 mmol) in 4 ml of acetone was added to a solution of 340 mg (13 mmol) of NH₂BrNphAc in 6 ml of acetone. The mixture was stirred for 4 h at 20°C, then the product was precipitated with cold water and recrystallized from 5 ml of ethanol. Yield: 200 mg (65%), m.p. 103°C, *R_F* 0.15. (Found: C, 52.60; H, 4.74; N, 10.71. C₁₁H₁₂N₂O₄ requires C, 52.33; H, 4.79; N, 11.11%.)

Pentamethylene-(4-amino-3-nitrophenacyl)sulphonium bromide (VI, Table 1). Thiacyclohexane (450 mg; 44 mmol) was added to a solution of 150 mg (6 mmol) of NH₂BrNphAc in 3 ml of acetone. The mixture was stirred for 4 h at 20°C. The precipitated solids were filtered off, washed with acetone and *n*-hexane, then dried. Yield: 170 mg (81%), m.p. 144°C, *R_F* 0.0. (Found: C, 43.01; H, 4.79; N, 7.81. C₁₃H₁₇N₂O₃Br requires C, 43.22; H, 4.74; N, 7.75%.)

α -Imidazolyl-4-amino-3-nitroacetophenone (VII, Table 1). NH₂BrNphAc [120 mg (5 mmol)] in 2 ml of acetone was added to a solution of 200 mg of imidazole (29 mmol) in 3 ml of acetone. After 20 h at 20°C the product was precipitated with water, filtered off and recrystallized from 5 ml of ethanol. Yield: 90 mg (75%), *R_F* 0.0. The substance decomposed on heating. (Found: C, 53.19; H, 3.93; N, 22.59. C₁₁H₁₀N₄O₃ requires C, 53.39; H, 4.08; N, 22.88%.)

Modification of pepsin with NH₂BrNphAc at different pH values. Portions (3 ml) of 0.3% pepsin solution in 0.2M-Na₂HPO₄/citric acid, pH 5.2, were adjusted to pH 2.5–5.2 with 6M-HCl. Then 0.3 ml portions of 0.3% NH₂BrNphAc solution in dimethylformamide (corresponding to 14-fold molar excess) were added. After 20 h at 37°C each mixture was passed through a Sephadex G-25 column (1.5 cm × 25 cm). NH₂BrNphAc incorporation was calculated from u.v. spectra of protein fractions. The following molar absorption coefficients were used for NH₂BrNphAc residues that have reacted with methionine residues: $\epsilon_{280} = 10\,700$, $\epsilon_{316} = 16\,300$; for pepsin, $\epsilon_{280} = 46\,000$.

No. of NH₂BrNphAc residues/pepsin molecule =

$$\frac{A_{316} \times 2.83}{(A_{280} - A_{316}) \times 0.657}$$

Reaction of aspergillopepsin A with NH₂BrNphAc. Aspergillopepsin A (3 mg in 3 ml of 0.2M-Na₂HPO₄/citric acid, pH 2.8) was treated with 0.3 ml of 0.3% NH₂BrNphAc solution in dimethylformamide. After 20 h at 20°C the mixture was passed through a column (1 cm × 33 cm) of Sephadex G-25. No changes in u.v. spectrum or activity of the enzyme were registered.

***NH₂BrNphAc* modification of H₂O₂-oxidized pepsin.** Pepsin solution in water (1%) was adjusted to pH 3.2 with HClO₄, then H₂O₂ was added to 0.3 M final concentration. The mixture was incubated for 70 min at 30°C (Kido & Kassel, 1975), then unchanged H₂O₂ was removed by gel filtration through an Acrllex P-10 column. To obtain a 0.3% solution of oxidized pepsin, 4 M-glycine/HCl, pH 5.2, was added to a final concentration of 0.2 M. Portions (3 ml) of this solution were removed and adjusted to pH 2.5–5.2 with 6 M HCl, then 0.3 ml portions of 0.3% NH₂BrNphAc solution were added, followed by 20 h incubation at 37°C. Gel filtration of the probes and the assay of NH₂BrNphAc incorporation were performed as described above.

***NH₂BrNphAc* modification of pepsin (preparative experiment).** NH₂BrNphAc (5 ml of a 0.3% solution in dimethylformamide) was added to a solution of 150 mg of pepsin in 50 ml of 0.2 M-Na₂HPO₄/citric acid, pH 2.8. After 20 h at 37°C the mixture was passed through a column (2.5 cm × 85 cm) of Sephadex G-25. Pepsin-containing fractions were pooled and freeze-dried. Incorporation of NH₂BrNphAc was found to be equal to 1.1–1.2 residues/protein molecule. The yield was 75 mg (50%).

Digestion of NH₂BrNphAc modified pepsin and isolation of labelled peptides. NH₂BrNphAc-modified pepsin (300 mg) was dissolved in 54 ml of 0.05 M-Tris/HCl buffer, pH 7.5; after 30 min, thermolysin (3 mg in 6 ml of water) was added and the mixture was left for 3 h at 37°C. The hydrolysate was fractionated on a column (3 cm × 140 cm) of Sephadex G-25 in water. Fractions containing coloured peptides were evaporated *in vacuo* and purified by paper electrophoresis (100 V/cm) and chromatography on Whatman 3MM paper. The following pyridine/acetic acid/water mixtures were used as buffer solutions for the electrophoresis: pH 5.6, 4:1:995; pH 6.5, 25:1:1974 (by vol.). Butanol/pyridine/water/acetic acid (15:10:3:12, by vol.) was used for paper chromatography. Coloured bands were cut out and eluted with water.

Structure determination of peptides. The amino acid sequence of peptides was carried out by the Edman procedure combined with dansylation of *N*-terminal amino acids; dansyl-amino acids were identified by two-dimensional t.l.c. on polyamide sheets (Woods & Wang, 1967). The amino acid composition of peptides was determined after 24 h hydrolysis with 5.7 M-HCl at 105°C on a Durrum D-500 amino acid analyser.

Results and Discussion

The coloured phenacyl bromide derivative α -bromo-4-amino-3-nitroacetophenone (IV) was synthesized as shown in Scheme 1. Acetylation

of *p*-aminoacetophenone gave *p*-acetamidoacetophenone (I), which was nitrated with HNO₃/acetic anhydride, giving 4-acetamido-3-nitroacetophenone (II) with 65% yield (Raadsveid, 1935). The latter ketone was treated with Br₂ in acetic acid, under conditions described for *p*-acetamidoacetophenone bromination (Gagin *et al.*, 1969), to yield previously undescribed α -bromo-4-acetamido-3-nitroacetophenone (III). Removal of the blocking acetyl group from this compound was achieved by brief heating with 24% HBr, which gave NH₂BrNphAc (IV) obtained with 81% yield.

To study the spectral characteristics of products of NH₂BrNphAc interaction with protein functional groups we synthesized derivatives with thioether, imidazole and carboxy groups corresponding to the structures formed after reaction with side chains of methionine, histidine, glutamic (and aspartic) acid respectively. Thiacyclohexane, imidazole and propionic acid gave with NH₂BrNphAc products with characteristic u.v. spectra (Table 1 and Fig. 1) useful for preliminary identification of protein groups reacting with NH₂BrNphAc under a given set of conditions.

The reaction of NH₂BrNphAc with porcine pepsin at pH 2.5–5.2 (molar ratio reagent/protein 14:1) and at 37°C gives a derivative that after its

Scheme 1. Synthesis of α -bromo-4-amino-3-nitroacetophenone

separation from excess reagent shows an absorption maximum at 280 nm (conventional protein maximum), 316 and 390 nm (Fig. 1). Two latter maxima are characteristic for the sulphonium salt (VI) (Table 1). This strongly suggests the formation of

methionine sulphonium salt in the course of pepsin reaction with $\text{NH}_2\text{BrNphAc}$ in acidic solutions:

Table 1. Absorption spectra of $\text{NH}_2\text{BrNphAc}$ and model compounds in water

Compound	λ_{max} (nm)	ϵ_M ($\text{M}^{-1} \cdot \text{cm}^{-1}$)
$\text{H}_2\text{N}-\text{C}_6\text{H}_3(\text{NO}_2)-\text{C}(=\text{O})-\text{CH}_2\text{Br}$ (IV)	270	13 600
	307	15 100
	400	4500
$\text{H}_2\text{N}-\text{C}_6\text{H}_3(\text{NO}_2)-\text{C}(=\text{O})-\text{CH}_2-\text{O}-\text{C}(=\text{O})-\text{C}_2\text{H}_5$ (V)	265	14 000
	307	12 700
	395	3000
$\text{H}_2\text{N}-\text{C}_6\text{H}_3(\text{NO}_2)-\text{C}(=\text{O})-\text{CH}_2-\text{S}^+(\text{CH}_2)_5\text{CH}_3 \text{ Br}^-$ (VI)	255	14 300
	316	16 300
	390	8000
$\text{H}_2\text{N}-\text{C}_6\text{H}_3(\text{NO}_2)-\text{C}(=\text{O})-\text{CH}_2-\text{N} \begin{array}{c} \diagup \diagdown \\ \diagdown \diagup \end{array}$ (VII)	265	14 800
	307	16 200
	395	3000

At pH 2.5–2.8, one $\text{NH}_2\text{BrNphAc}$ residue is incorporated into pepsin molecule. The pH shift from 2.5 to 5.2 is accompanied by steady increase in $\text{NH}_2\text{BrNphAc}$ incorporation [from one to two residues (Fig. 2)]. The attachment of one $\text{NH}_2\text{BrNphAc}$ residue at pH 2.5 results in a 45% loss of proteolytic activity when measured with haemoglobin as a substrate. Further increase in $\text{NH}_2\text{BrNphAc}$ incorporation causes no significant change in the enzyme activity.

To confirm that $\text{NH}_2\text{BrNphAc}$ interacts with methionine residues in porcine pepsin, we checked the action of this reagent on the enzyme oxidized with H_2O_2 at pH 3.2. According to Kido & Kassel (1975) this treatment converts into sulfoxides two and a half from four methionine residues present in porcine pepsin. The activity of oxidized pepsin corresponded to 82% of that of the native enzyme; no significant changes in K_m and K_{cat} values for hydrolysis of peptide substrates were noted.

Oxidized pepsin prepared by us did not react with $\text{NH}_2\text{BrNphAc}$ at pH values lower than 3, thus confirming the involvement of methionine into modification of proteins by $\text{NH}_2\text{BrNphAc}$. On the other hand $\text{NH}_2\text{BrNphAc}$ incorporation was ob-

Fig. 1. Absorption spectra of $\text{NH}_2\text{BrNphAc}$, model compounds and $\text{NH}_2\text{BrNphAc}$ -treated pepsin in water 1, $\text{NH}_2\text{BrNphAc}$ (IV); 2, α -propionyloxy-4-amino-3-nitroacetophenone (V); 3, pentamethylene-(4-amino-3-nitrophenacyl)sulphonium bromide (VI); 4, pepsin, modified with $\text{NH}_2\text{BrNphAc}$ at pH 2.8; 5, oxidized pepsin, modified with $\text{NH}_2\text{BrNphAc}$ at pH 5.2.

Fig. 2. pH-dependence of the incorporation of $\text{NH}_2\text{Br-NphAc}$ into pepsin

Pepsin solution (0.3%) was incubated with 14-fold molar excess of $\text{NH}_2\text{BrNphAc}$ for 20 h at 37°C . For details, see the text. O, Native pepsin; ●, pepsin oxidized with H_2O_2 .

served when the pH of reaction mixture was increased from 3 to 5.6 (Fig. 2), and it attained a value of 0.95 residue per molecule of oxidized pepsin. This derivative showed in its u.v. spectrum a shoulder at 307 nm characteristic of carboxy-group esterification or histidine alkylation. Its activity was equal to 75% of that of the starting preparation of oxidized pepsin. Obviously some other group, most likely the ω -carboxy group of dicarboxylic amino acids reacted at pH higher than 3. The effect of this reaction on pepsin activity is apparently not very significant. Anyway, it is clear that modification of pepsin at pH values lower than 3, under conditions comparable with that used for modification by *p*-bromophenacyl bromide, is directed towards a methionine residue and is accompanied by substantial decrease in enzyme activity. Since the structure of $\text{NH}_2\text{BrNphAc}$ is not identical with that of *p*-bromophenacyl bromide, the derivative previously used for pepsin modification, we considered it necessary to compare the action of both reagents on pepsin. It happened that pepsin modified with

Fig. 3. Fractionation of a thermolysin digest of $\text{NH}_2\text{BrNphAc}$ -treated pepsin (300 mg) on a column ($3\text{ cm} \times 140\text{ cm}$) of Sephadex G-25 (superfine grade) —, A_{316} ; ----, A_{280} .

(molar ratio enzyme/*p*-bromophenacyl bromide/ $\text{NH}_2\text{BrNphAc}$ 1:14:14) at pH 2.8, the incorporation of $\text{NH}_2\text{BrNphAc}$ was 0.6 residue per pepsin molecule, i.e., almost half the incorporation in the absence of *p*-bromophenacyl bromide. This experiment suggested that both reagents interact with the same functional groups of the enzyme.

In order to localize the groups modified by $\text{NH}_2\text{BrNphAc}$, the pepsin derivative prepared by the reaction with this inhibitor at pH 2.8 was digested by thermolysin at pH 7.5. Separation of the hydrolysate on Sephadex G-25 gave three coloured fractions (Fig. 3). Fraction III contained the inhibitor detached from the peptides. Fractions I and II were submitted to extensive purification by paper chromatography and electrophoresis. From the fraction II, pure peptide A, containing the coloured inhibitor, was isolated (Table 2). Its amino acid composition as well as the sequence of the first five residues from the *N*-terminus revealed that this peptide corresponded to the pepsin sequence from residues 287 to 294.

Pepsin sequence:

Thr-Phe-Glu-Gly-Met-Asp-Val-Pro-Thr-Ser-Ser-Gly-Glu-Leu

Peptide A:

Phe-Glu-Gly-Met-Asp, Val, Pro, Thr

Peptide B:

Phe-Glu-Gly-Met-Asp, Val, Pro, Thr, Ser, Ser, Gly, Glu

p-bromophenacyl bromide at pH 2.8 did not react with $\text{NH}_2\text{BrNphAc}$ under the same set of conditions. When pepsin was treated with equimolar mixture of *p*-bromophenacyl bromide and $\text{NH}_2\text{BrNphAc}$

Coloured peptide B, isolated from fraction I according to its amino acid composition and *N*-terminal sequence shown above comprised the stretch of pepsin sequence between residues 287 and 298

Table 2. *Amino acid composition of coloured peptides*
The composition is given in nmol per sample of hydrolysate. The numbers in parentheses are nearest integers, taking glutamic acid as $\equiv 1$ for peptide A, phenylalanine $\equiv 1$ for peptide B, aspartic acid $\equiv 1$ for peptide C. Abbreviation used: Met-SO₂, methionine sulphone.

Amino acid	Composition		
	Peptide A	Peptide B	Peptide C
Asp	8.3 (1)	2.1 (1)	43.0 (1)
Met-SO ₂	3.7 (1)	0.5 (1)	—
Thr	7.5 (1)	2.4 (1)	37.6 (1)
Ser	—	5.8 (2)	—
Glu	7.6 (1)	7.8 (2)	—
Pro	7.0 (1)	5.5 (1)	—
Gly	8.3 (1)	8.9 (2)	—
Val	7.8 (1)	4.4 (1)	44.7 (1)
Phe	7.9 (1)	3.3 (1)	43.2 (1)
Arg	—	—	23.2 (1)

(Table 2). Both peptides A and B showed absorption maxima at 316 nm, indicating the attachment of NH₂BrNphAc to the methionine-290 residue. In the course of Edman degradation, the modified methionine-290 residue appeared after dansylation as three spots on chromatograms. One of them corresponded to methionine, two others being presumably the dansyl derivatives of homoserine and of the product of methyl-group detachment from the sulphonium salt (VIII).

One more labelled peptide (peptide C) was isolated from fraction II. Peptide C showed an absorption maximum at 307 nm, which suggested the modification of a carboxy group. The amino acid composition of this peptide corresponded fairly well to the sequence Thr-Val-Phe-Asp-Arg (residues 312–316) of pepsin. The isolation of this peptide clearly indicates the modification by NH₂BrNphAc of the aspartic acid-315 residue.

It has to be stressed that in the thermolysin hydrolysate of pepsin inactivated by NH₂BrNphAc at pH 2.8, 97% of the coloured label was in methionine-containing peptides A and B, whereas only 2.5% was in peptide C. This clearly shows almost exclusive modification of the methionine-290 residue in pepsin under the conditions specified above. Hence one is induced to assume that the modification of porcine pepsin with *p*-bromophenacyl bromide also affects the methionine-290 residue. The previous assumption that it is the carboxy group of an aspartic acid residue that interacts with phenacyl halides causing partial inactivation of pepsin has to be rejected. As the present study shows, only marginal modification of

aspartic acid-315 proceeded at pH 2.8, i.e. under conditions used for pepsin inactivation by these reagents. More extended modification of this, or eventually other, carboxy groups was observed at higher pH, but no significant effect on pepsin activity was observed.

Substantial inactivation of porcine pepsin with NH₂BrNphAc is due to modification of methionine-290 with this reagent. Clearly methionine cannot be directly involved in the pepsin catalytic mechanism, since practically complete blockage of the residue did not lead to complete inactivation of the enzyme; on the contrary, substantial activity is conserved. It was found previously that the modification of pepsin with *p*-bromophenacyl bromide at pH 2.8 and 37°C did not change the k_{cat} value for substrate hydrolysis, but merely increased the K_m 2-fold (Erlanger *et al.*, 1967; Valueva *et al.*, 1971). These data are in line with the assumption that the reaction does not destroy the enzyme catalytic centre but rather that it modified the substrate-binding site. Another confirmation of this conclusion was provided by the fact that *p*-bromophenacyl bromide-modified pepsin reacted with specific inactivators of carboxylic proteinases, namely diazocarbonyl compounds (Erlanger *et al.*, 1967), epoxides (Tang, 1971) and pepstatin (Kunimoto *et al.*, 1974). Furthermore, the modified pepsin remains as active as the native enzyme in transpeptidation reactions (Valueva *et al.*, 1971).

The inhibitory action of phenacyl bromides might be rationalized on the basis of pepsin tertiary structure. It has been shown that porcine pepsin as well as other pepsin-like carboxylic proteinases possess a pronounced cleft in their tertiary structures that harbours the active site of these enzymes (Andreeva *et al.*, 1978; Hsu *et al.*, 1977; Subramanian *et al.*, 1977; James *et al.*, 1977). As observed in the pepsin model built by Andreeva *et al.* (1978), methionine-290 is situated on the edge of the cleft, overlooking its entrance. It might be assumed that the rather bulky inhibitor residue attached to methionine-290 can at least partially shield the entrance to the cleft, thus creating substantial steric hindrance to the approaching substrate. It is clear that the extent of this hindrance and, as a consequence, of pepsin inactivation, has to be dependent on the size of the inhibitor as well as on the size of the substrate. This explains marked variations in the degree of pepsin inactivation achieved with differently substituted phenacyl halides (Erlanger *et al.*, 1965).

This explanation of the inhibitory effects of phenacyl halides on porcine pepsin appears to be consistent with the data on the action of these reagents on various carboxylic proteinases. The dependence of the reaction on the presence of suitably located methionine residues allows us to

explain previously inexplicable data on the reaction specificity of different pepsins.

p-Bromophenacyl bromide has been shown to cause 70–80% inactivation of human pepsin (Hunkapiller *et al.*, 1970) and monkey pepsins (Kageyama & Takahashi, 1976). A methionine residue had been found in the 290-position of human pepsin (Sepulveda *et al.*, 1975b), and this explains the inactivation of this enzyme. No structural data are available for monkey pepsins, but it might be assumed that sequence differences from human enzyme should not be many. No structural data are available, and hence no explanation can be given, for the inactivation of carboxylic proteinase from *Rhodotorula glutinis* (Oda *et al.*, 1972) or for that from human seminal plasma (Ruenwongsa & Chulavatnotol, 1975). On the other hand, in calf chymosin, the 290-position is occupied by glutamic acid (Foltmann *et al.*, 1977), and this enzyme was found to be resistant to *p*-bromophenacyl bromide inactivation (Chang & Takahashi, 1973; Cheesemann, 1969). The same is true for penicillopepsin (asparagine-290) (Sodek & Hoffmann, 1968; Hsu *et al.*, 1977) and aspergillopepsin A, which contains no methionine residues (Lobareva *et al.*, 1972). The latter enzyme was shown by us not to interact with $\text{NH}_2\text{BrNphAc}$ at pH 2.8. Human gastricsin (Hunkapiller *et al.*, 1970), chicken pepsin (Lokshina *et al.*, 1972; Llewellyn & Green, 1975), cathepsin D (Keilova, 1970) and a number of carboxylic proteinases from fungi [*Cladosporium* sp. (Oda *et al.*, 1973), *Mucor miechi* (Sterberg, 1972), *Penicillium duponti* (Eni *et al.*, 1976), *Rhizopus chinensis* (Mizobe *et al.*, 1973)] are also non-susceptible to inactivation by *p*-bromophenacyl bromide. No structural data are available for most of these enzymes, but one must bear in mind that fungal carboxylic proteinases contain only a few, if any, methionine residues.

Thus the study of pepsin modification with the coloured reagent $\text{NH}_2\text{BrNphAc}$ showed that at pH values lower than 3 this reagent, as well as presumably other phenacyl halides, modified the methionine-290 residue. The residues of dicarboxylic amino acids are likely to react at higher pH.

The type of inhibition achieved by phenacyl halides in the case of porcine pepsin deserves few comments. X-ray analysis of a number of enzymes revealed that many of them are characterized by the presence of more or less pronounced cleft, where substrate molecules become bound. Evidently the attachment of any bulky substituent on the edge of this cleft will result in substantial steric hindrance to the substrate approaching the thus-shielded active site. It appears that inhibition of this type, which might be termed 'substrate-approach inhibition' could be rather widely distributed.

The experience gained in the course of the present

study has showed that the coloured phenacyl bromide derivative $\text{NH}_2\text{BrNphAc}$ might be quite useful for the chemical modification of various proteins.

References

- Andreeva, N. S., Fiodorov, A. A., Gushina, A. E., Riskulov, R. R., Shutzkever, N. E. & Safro, M. G. (1978) *Mol. Biol. (Moscow)* **12**, 922–936
- Anson, M. D. (1938) *J. Gen. Physiol.* **22**, 79–83
- Bruining, W. I. (1922) *Rec. Trav. Chim. Pay-Bas* **41**, 655–686
- Chang, W. J. & Takahashi, K. (1973) *J. Biochem. (Tokyo)* **74**, 231–237
- Cheesemann, G. C. (1969) *J. Dairy Res.* **36**, 299–312
- Diopoli, I. & Olomucki, M. (1972) *Biochim. Biophys. Acta* **263**, 220–225
- Eni, S., Myera, D. W. & Lacobucci, G. A. (1976) *Biochemistry* **15**, 842–848
- Erlanger, B. F., Wratsanos, S. M., Wassermann, N. & Cooper, A. G. (1965) *J. Biol. Chem.* **240**, 3447–3448
- Erlanger, B. F., Wratsanos, S. M., Wassermann, N. & Cooper, A. G. (1966) *Biochem. Biophys. Res. Commun.* **23**, 243–245
- Erlanger, B. F., Wratsanos, S. M., Wassermann, N. & Cooper, A. G. (1967) *Biochem. Biophys. Res. Commun.* **28**, 203–208
- Foltmann, B., Redersen, V. B., Jacobsen, D. H., Kauffmann, D. & Wybrandt, G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2321–2324
- Gagin, F., Csavassy, G. H. & Bebesel, E. (1969) *J. Prakt. Chem.* **311**, 168–170
- Glover, G. I., Marino, P. S. & Petersen, I. R. (1976) *Biochemistry* **15**, 3754–3760
- Gross, E. & Morrel, G. L. (1966) *J. Biol. Chem.* **241**, 3638–3648
- Hsu, I.-N., Delbaere, L. T. F., James, M. N. G. & Hoffmann, T. (1977) *Nature (London)* **266**, 140–145
- Hunkapiller, M., Heinzer, I. E. & Mills, I. N. (1970) *Biochemistry* **9**, 2897–2902
- James, M. N. G., Hsu, I.-N. & Delbaere, T. J. (1977) *Nature (London)* **267**, 808–813
- Kageyama, T. & Takahashi, K. (1976) *J. Biochem. (Tokyo)* **79**, 455–469
- Keilova, H. (1970) *FEBS Lett.* **6**, 312–314
- Kido, K. & Kassel, B. (1975) *Biochemistry* **14**, 631–635
- Kovaleva, G. G., Jusupova, M. P., Balandina, G. N., Lisogorskaya, E. N. & Stepanov, V. M. (1977) *Biokhimiya* **42**, 534–539
- Kunimoto, S., Aoyagi, T., Nishsawa, R., Konai, T., Takushi, T. & Umezawa, H. (1974) *J. Antibiot. Ser. B* **27**, 413–418
- Llewellyn, I. M. & Green, M. L. (1975) *Biochem. J.* **151**, 319–326
- Lobareva, L. S., Kovaleva, G. G., Shimanskaya, M. P. & Stepanov, V. M. (1972) *Biokhimiya* **37**, 198–207
- Lokshina, L. A., Dyuklova, T. A. & Orekhovich, V. N. (1972) *Biokhimiya* **37**, 275–282
- Mizobe, F., Takahashi, K. & Ando, T. (1973) *J. Biochem. (Tokyo)* **73**, 61–68
- Oda, K., Kamada, M. & Murao, S. (1972) *Agric. Biol. Chem.* **36**, 1103–1108

- Oda, K., Funakoshi, S. & Murao, S. (1973) *Agric. Biol. Chem.* **37**, 1723-1729
- Raadsveid, C. W. (1935) *Rec. Trav. Chim. Pays-Bas* **54**, 811-827
- Ruenwongsa, P. & Chulavatnotol, M. (1975) *J. Biol. Chem.* **250**, 7574-7578
- Sepulveda, P., Mareiniszyn, J., Lin, D. & Tang, J. (1975a) *J. Biol. Chem.* **250**, 5082-5088
- Sepulveda, P., Jackson, K. W. & Tang, J. (1975b) *Biochem. Biophys. Res. Commun.* **63**, 1106-1112
- Sodek, I. & Hoffmann, T. (1968) *J. Biol. Chem.* **243**, 450-451
- Stepanov, V. M. & Vaganova, T. I. (1969) *Biokhimiya* **28**, 540-546
- Sterberg, M. (1972) *Biochem. Biophys. Acta* **285**, 383-393
- Subramanian, E., Swan, I. D. A., Davies, D. R., Jenkins, J. A., Tickle, I. J. & Blundell, T. L. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 556-559
- Tang, J. (1971) *J. Biol. Chem.* **240**, 4510-4517
- Valueva, T. A., Ginodman, L. M. & Terekhova, S. E. (1971) *Biokhimiya* **36**, 667-673
- Woods, K. R. & Wang, K.-T. (1967) *Biochim. Biophys. Acta* **133**, 369-370