CHEMICAL MODIFICATION AS A METHOD OF STUDYING THE FUNCTIONAL GROUPS OF CARBOXYLIC PROTEINASES

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A review of the literature on the chemical modification of the functional groups of carboxylic proteinases is given. The following branches of the subject are considered: spatial organization and the modification of carboxy groups and amino groups of arginine, histidine, tyrosine, tryptophan, methionine, cystine, and cysteine residues. Chemical modification is considered as a method of studying the functional groups of enzymes which, in association with the results of x-ray structural analysis, permits an idea to be obtained of the mechanism of the action of carboxylic proteinases.

Carboxylic, or acid, proteinases — proteolytic enzymes that are active in an acid medium — are widely distributed in nature and are found in animals, plants, and microorganisms. Well-known representatives of this class are the enzymes of the digestive tract of mammals — pepsin, chymosin, and gastricsin. Carboxylic proteinases were the first enzymes that were isolated and used by man [1]. Their wide use in practice explains the increased interest in the study of this group of enzymes [2-5]. As a rule, carboxylic proteinases have molecular weights of about 35,000 and consist of a single polypeptide chain containing about 325 amino acid residues. A characteristic feature of them is a high content of acidic and a low content of basic amino acids. They include approximately equal numbers of hydrophobic and aromatic amino acids. The substrate specificity of the carboxylic proteinases is broad. They all hydrolyze the bonds formed by hydrophobic amino acids while, for example, serine proteinases exhibit substantial differences in substrate specificity.

The complete amino acid sequences of porcine pepsin [6], bovine chymosin [7], and penicillopepsin [8] and partial sequences of a number of other proteinases [9-13] have been established. A comparison of primary structures reveals a high homology of the sequences. Thus, in porcine pepsine and penicillopepsin the identical residues make up 32% of the total number of amino acids and form 11 sections with a high degree of homology [14]. The amino acid sequences around the catalytically important aspartic acid residues are very close or identical in all known cases.

Spatial Organization. X-Ray structural analyses have recently been performed on a number of proteinases; porcine pepsin [15], penicillopepsin [14], *Rhizopus* pepsin [16], and *Endothia* pepsin [16]. It was found that even the tertiary structures of proteinases isolated from such different sources are homologous [17]. The molecule has dimensions of 65 × 49 × 39 Å (for penicillopepsin) and consists of two globular domains connected by a short section of polypeptide chain with an extended cleft between the domains. The cleft contains a considerable number of hydrophobic amino acids and is accessible to a solvent. The catalytically active aspartic acid residues 32 and 215 are located in it. In the N-terminal domain there is a hydrophobic "pocket," including the Trp-39, Trp-71, and Tyr-75 residues. For *Rhizopus* pepsin it has been shown [8] that pepstatin — a polypeptide hydrophobic inhibitor of carboxylic proteinases — is bound in this cleft. The extent of the cleft — about 30 Å — agrees well with results showing that 7-8 amino acid residues of the substrate are bound in the active center of a carboxylic proteinase.

The catalytically active aspartic acid residues 32 and 215 are located so close to one another that the existence of a hydrogen bond and sharing of the proton between them are possible [19]. Analysis of the pH dependence of the activity of porcine pepsin has shown that there are two carboxy groups in its catalytic center. It is assumed that Asp-215 has the high pK_a value of 4.5 and functions in the protonated form. The Asp-32 carboxyl with pK_a = 1.5 is strongly acidic and participates in catalysis in the dissociated form. Such a low

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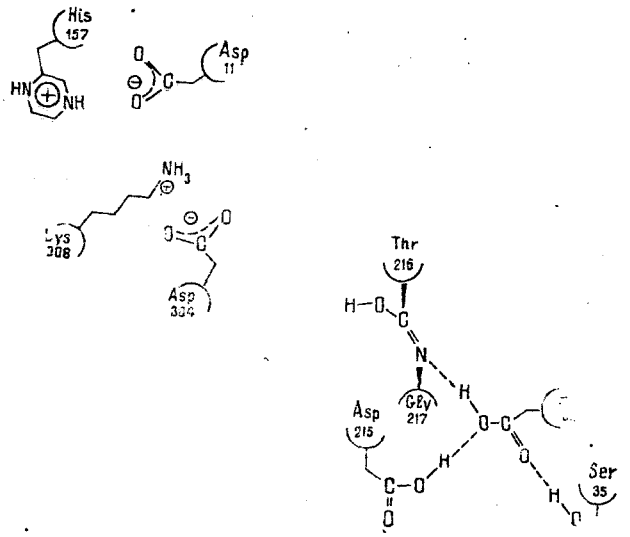


Fig. 1. Suggested charge-transfer system increasing the electrophilicity of the proton between Asp-215 and Asp-32 [19].

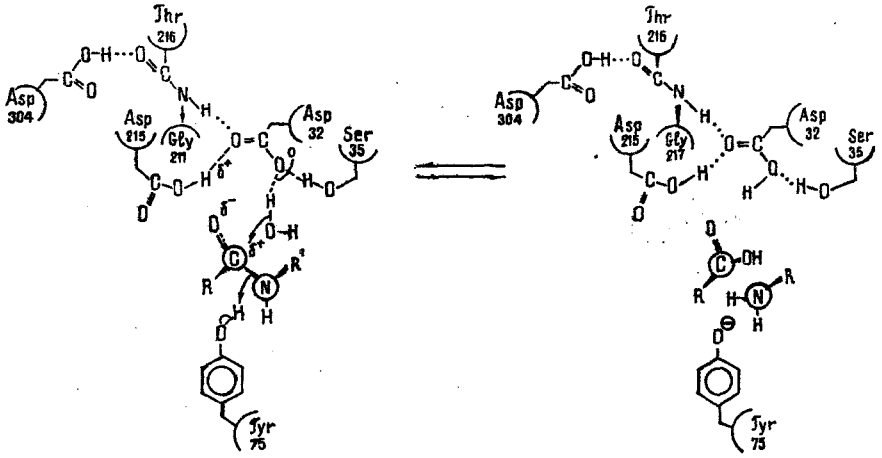


Fig. 2. Suggested mechanism of the section of carboxylic proteinases [19].

pK_a value is probably connected with the peculiar environment of this group. An analysis of the tertiary structure of penicillopepsin led James et al. [19] to assume that the molecule contained a charge-transfer chain ensuring an increase in the electrophilicity of the proton shared between the Asp-32 and Asp-215 residues (Fig. 1). The Asp-304 residue forms an ion pair with Lys-308. Close to this pair is the His-157 and Asp-11 ion pair. When a substrate is bound, conformational changes take place which lead to the formation of a new ion pair, between Asp-11 and Lys-308, and the liberation of Asp-304. Through the charge-transfer system, the latter gives rise to an increase in the electrophilicity of the shared proton. All these amino acid residues are invariant in pepsin and penicillopepsin (with the exception of His-157). Both catalytically active carboxy groups are, as the authors emphasize, relatively inaccessible for the substrate molecule and therefore conformational changes must take place during the binding of the substrate. Thus, on the interaction of penicillopepsin with the inhibitor 1,2-epoxy-3-p-nitrophenoxypropane the position of Tyr-75 changes and this residue, previously connected by a hydrogen with Trp-39, shifts to a position coplanar with the p-nitrophenoxy group of the epoxide bound to Asp-32. It is interesting that this change is similar to the rearrangement that takes place on the binding of a substrate with carboxypeptidase A. A scheme of the mechanism of the action of carboxylic proteinases has been based on the results of x-ray structural analysis (Fig. 2) [19].

Table 1. Functional Roles of Individual Amino Acids in Porcine Pepsin Revealed by Chemical Modification

Residue No.*	Amino Acid	Modifying agent	Assumed role	Literature
1	Isoleucine	Ethoxyformic anhydride	Functionally unimportant	95
1 (320)		Nitrous acid		94
1 (320)		Ketene		93
1 (320)		Pyridoxal phosphate		97
9 (174)	Tyrosine	Iodine	Binding of substrate	116
9 (174, 189)		tetranitromethane		113
32 (215)	Aspartic acid	1,2-Epoxy-3-p-nitro-phenoxypropane	In active center	69-71
39	Tryptophan semicysteine	2-Hydroxy-5-nitrobenzyl bromide	Structural role	119
45	Hemicysteine	Mercaptoethanol	Structural role	127,128
50	"	"	"	"
53	Histidine	Ethoxyformic anhydride	Inaccessible to modification	95
68	Phospho-serine	Phosphatase	Functionally unimportant	130
80 (199, 246)	Methionine	Hydrogen peroxide	Binding of substrate	121
141 (181)	Tryptophan	2-Hydroxy-5-nitrobenzyl bromide	Structural role	119
174 (9)	Tyrosine	Iodine	Binding of substrate	116
174 (9, 189)		tetranitromethane		113
181 (141)	Tryptophan	2-Hydroxy-5-nitrobenzyl bromide	Structural role	119
189	Tyrosine	p-Benzenediazonium chloride	Binding of substrate	113
189 (9, 174)		tetranitromethane		113
190	Tryptophan	2-Hydroxy-5-nitrobenzyl bromide	Structural role	119
199 (80, 246)	Methionine	Hydrogen peroxide	Binding of substrate	121
206	Hemicystine	Mercaptoethanol	Readily accessible to modification	126, 127
210	"	"		"
215	Aspartic Acid	Diazocarbonyl compounds	In active center	22-32
243 (298, 327)	"	Mono-DNP-hexamethylene-diamine	In catalysis	83, 84
246 (80, 199)	Methionine	Hydrogen peroxide	Binding of substrate	121
250	Hemicystine	Mercaptoethanol	Functionally unimportant	126, 127
283	"	"	"	"
290	Methionine	1,2-Epoxy-3-p-nitro-phenoxypropane	Binding of substrate	71
290		4-Amino-α-bromo-3-p-nitroacetophenone		124

Table 1 (continued)

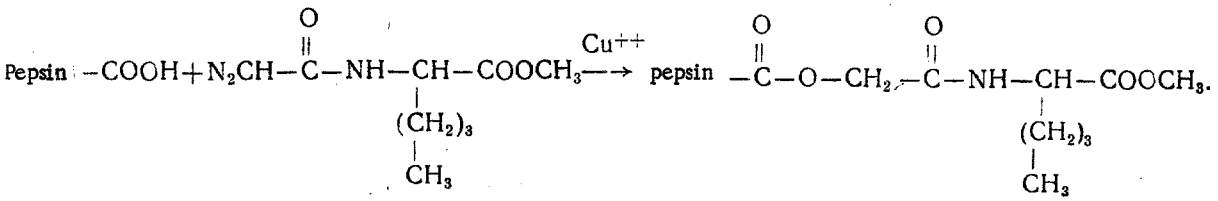
Residue No.*	Amino Acid	Modifying agent	Assumed role	Literature
290 (243, 327)	Glutamic acid	Mono-DNP-hexamethylene-diamine	Participation in catalysis	83, 84
300	Tryptophan	2-Hydroxy-2-nitrobenzyl bromide	Inaccessible to modification	119
308	Arginine	Phenylglyoxal	"	102
308		Butane-2,3-dione		103
316	"	Phenylglyoxal	Participation in catalysis	102
		Butane-2,3-dione	"	103
320	Lysine	S-Carboxymercaptide of p-mercuribenzoic acid	Functionally unimportant	96
320 (1)		Ketene		93
320 (1)		Nitrous acid		94
320 (1)		Pyridoxal phosphate		97
327 (243, 298)	Alanine	Mono-DNP-hexamethylene-diamine	Participation in catalysis	83, 4

*The numbers of other residues reacting under the same conditions are given in parentheses.

At the present time, a large amount of material on the chemical modification of individual functional groups of various enzymes has been accumulated, but the most complete information exists only for porcine pepsine. Details of the modification and functional roles of individual amino acid residues are given in Table 1.

Modification of Carboxy Groups, Diazo Compounds. The reactions of porcine pepsin with diazomethane [20] and diazodiphenylmethane [21] take place insufficiently specifically. Diazocarbonyl compounds have proved to be specific inhibitors of carboxylic proteinases.

In 1966, Rajagopolan et al. [22] found that in the presence of ions of bivalent copper the methyl ester of diazoacetyl-D,L-norleucine (DAN) inhibited pepsin at pH 5.6 and 14°C. In this process, one inhibitor residue adds to the enzyme molecule. The authors assumed that esterification of a carboxy group took place in accordance with the reaction



The inclusion of the inhibitor is conveniently determined from the amount of norleucine in an acid hydrolysate of the protein. Pepsinogen, and also pepsin inactivated at pH 8.0, does not react with DAN. The specific reaction with pepsin takes place only in the presence of copper ions. When the enzyme was treated with a 100- to 500-fold excess of the inhibitor in a solution containing no copper ions, there was only 60-70% inactivation after 5-6 h, 1.5-3.5 inhibitor residues having been incorporated.

In recent years, a number of compounds has been suggested which inhibited pepsin in the presence of copper ions: derivatives of diazoacetamide of the general formula $N_2CH{-}CO{-}CH{-}R_1$, diazoketones $N_2CH{-}CO{-}R_2$, and diazoesters.

Diazocarbonyl Compounds as Inhibitors of Porcine Pepsin

Derivatives of Diazoacetomide ($N_2CHCONHR_1$)

$N_2CHCONHCHCOOCH_3$ (with $CH_2)_3CH_3$ on the CH) — methyl ester of diazoacetylnorleucine (DAN) [22]

$N_2CHCONHCH_2COOC_2H_5$ — ethyl ester of diazoacetylglycine [23]

$N_2CHCONHCHCOOC_2H_5$ (with $CH_2C_6H_5$ on the CH) — ethyl ester of diazoacetylphenylalanine [23]

$N_2CHCONHCH_2CH_2NHC_6H_3(NO_2)_2$ — N-diazoacetyl-N'-DNP-ethylenediamine [24]

$N_2CHCONHCHCOOCH_3$ (with $(CH_2)_3NHC_6H_3(NO_2)_2$ on the CH) — methyl ester of α-N-diazoacetyl-δ-N'-DNP-ornithine [24]

$N_2CHCONHCHCOOCH_3$ (with $(CH_2)_3CH_3$ on the CH) — methyl ester of diazoacetylnorvaline [25]

Diazoketones (N_2CHCOR_2)

$N_2CHCOCHCH_2C_6H_5$ (with $NHSO_2C_6H_4CH_3$ on the CH) — 1-diazo-4-phenyl-3-tosylaminobutanone [23]

$N_2CHCOCH_2NHC_6H_3(NO_2)_2$ — 1-diazo-3-DNP-aminopropanone [26]

$N_2CHCOCH_2CH_2C_6H_5$ — 1-diazo-4-phenylbutan-2-one [27]

$N_2CHCOCHCH_2C_6H_5$ (with $NHCOOCH_2C_6H_5$ on the CH) — 3-benzyloxycarbonylamino-1-diazo-4-phenylbutanone (benzyloxycarbonylphenylalanyldiazomethane) [28]

$N_2CHCOC_6H_4Br$ — p-bromo-α-diazoacetophenone [29]

$N_2CHCOC_6H_5$ — benzoyldiazomethane [26]

Diazoesters

$N_2CHCOOCH_3$ — methyl diazoacetate [30]

$N_2CHCOOC_2H_5$ — ethyl diazoacetate [30]

$N_2CCOOC_2H_5$ (with $CH_2C_6H_4OH$ on the C) — ethyl α-diazo-β-p-hydroxyphenylpropionate [26]

Bifunctional compounds

$N_2CHCOCHCH_2C_6H_5$ (with $COCHN_2$ on the CH) — 1,1-bisdiazoacetyl-2-phenylethane [31]

$N_2CHCOCHBrCH_2C_6H_5$ — 1-bromo-1-diazoacetyl-2-phenylethane [31]

Radioactive and colored compounds have been used to estimate the inclusion of inhibitors in proteins. The dinitrophenyl label in N-diazoacetyl-N'-DNP-ethylenediamine [24] permits the the determination of the inclusion of the inhibitor by spectral methods, which considerably simplifies the investigation.

The capacity of diazoacetamide derivatives for inhibiting pepsin does not depend on the structure and sterochemistry of the radical R_1. The L and D isomers of the ethyl ester of

diazoacetylphenylalanine react with pepsin at the same rate. In spite of substantial differences in the structure of the radical R_1, the ethyl esters of diazoacetylglycine and diazoacetylphenylalanine inhibit pepsin at comparable rates. Conversely, inhibition by diazoacetamide derivatives depends substantially on the sterochemistry and structure of the radical R_2. In 10 minutes, the L isomer of 1-diazo-4-phenyl-3-tosylaminobutanone inactivates pepsin by 80%, and the D isomer by only 6% [23]. Complete inhibition of the enzyme by 1-diazo-4-phenylbutan-2-one, a close structural analog of phenylalanine, takes place with only a 1- to 2-fold excess of the inhibitor and a low concentration of copper ions [27]. 3-Benzyloxycarbonylamino-1-diazo-4-phenylbutanone inhibits pepsin completely without the addition of copper ions, although the latter accelerate this reaction [28]. The differences in the structural and stereochemical requirements for diazocarbonyl inhibitors of pepsin can be explained by comparing their structures with those of the peptide substrates of pepsin. Pepsin hydrolyzes peptide bonds formed by residues of hydrophobic amino acids. It is assumed that the zone of binding of the substrate in the enzyme molecule consists of a section A, interacting with the amino acid, the carboxy group of which forms the bond that is being attacked, and a section B, interacting with the amino acid the amino group of which takes part in this bond. We assume that the reaction of diazocarbonyl compounds with pepsin is preceded by an interaction with the substrate-binding zone in which side chains participate. Then the substituent R_2 in the diazoketones occupies section A (Fig. 3) and the residue R_1 in the diazoacetamide derivatives occupies section B. It may be assumed that the binding of the inhibitors in section A sterically promotes their interaction with the carboxy group of the enzyme, and therefore for inhibitors interacting with this section more or less pronounced requirements on the structure and stereochemistry are characteristic. Conversely, binding in section B, if it is realized at all, leads to an orientation of the diazo group on the inhibitor which is unfavorable for reaction with pepsin. Apparently, inhibitors that are derivatives of diazoacetamide react with the enzyme without preliminary binding in section B but because of this are insensitive to the structure and stereochemistry of the substituent R_1. Of course, the hypotheses put forward have only a preliminary nature and a systematic investigation of whole series of inhibitors is necessary to confirm them.

Diazocarbonyl compounds — both diazoketones [32] and diazoacetamide derivatives [33] — react with the same aspartic acid residue, occupying position 215 in porcine pepsin. Lundblad and Stein [30] have made a detailed investigation of the reaction of pepsin with diazo-

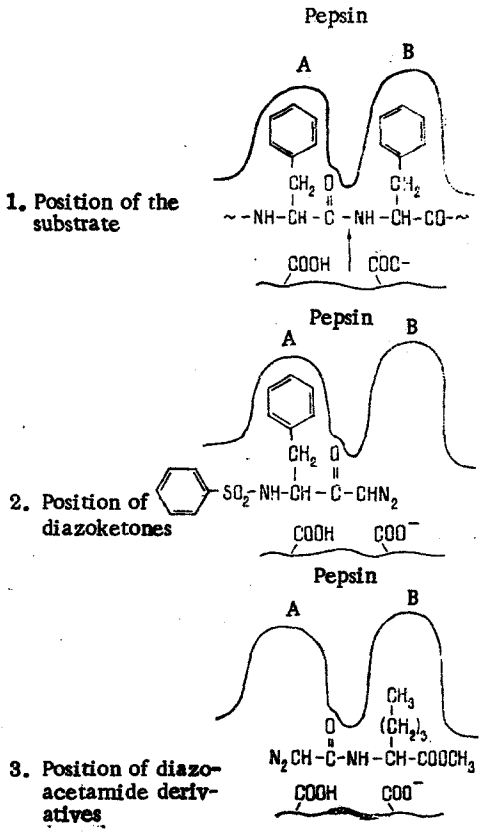


Fig. 3. Interaction of diazocarbonyl compounds with the substrate-binding zone in pepsin (hypothesis). The arrow shows the position of the bond undergoing attack in the substrate.

carbonyl compounds and have found that previous mixing of the inhibitor with copper ions accelerates the reaction. Apart from copper ions, only silver ions possess a catalytic action. A mechanism of the reaction has been put forward according to which the reaction of the diazocarbonyl compound with copper ions forms a carbene complex which then takes part in a reaction with the protein: $R{-}CO{-}CHN_2 + Cu^{++} \rightarrow R{-}CO{-}CH = Cu^{++} + N_2$. The complex can interact electrostatically with an ionized carboxy group of the protein and orient the intermediate compound for reaction with the protonated carboxy group. In confirmation of this hypothesis, the authors performed the reaction of pepsin with dimethylsulfonium phenacylide, for which the formation of a copper-carbene complex has been demonstrated [34].

Inactivation by diazocarbonyl compounds does not apparently cause irreversible changes in the conformation of the molecule, since complete reactivation is possible. Thus, porcine pepsin inactivated by N-diazoacetyl-N'-DNP-ethylenediamine or by diazoacetyl-3-DNP-aminopropan-2-one recovers its activity completely in 24 h at pH 4.5 and 20°C [35]. Complete restoration of activity is also observed for chicken pepsin [39].

The reaction with diazocarbonyl compounds is a general one for all carboxylic proteinases. Diazocarbonyl compounds inactivate the pepsin of man [37, 38], monkeys [39], cattle [40], and chickens [36, 41], porcine pepsin C [42], human gastricsin [37, 38], chymosin [36, 43], the cathepsins D from spleen [44, 45], from the spinal chord [46], from the adrenal glands [45], from the thyroid gland [47], and from skeletal muscle [48], cathepsin E [46], remmin [49], the proteinases of insectivorous plants [48, 50], and the proteinases of the mold fungi *Acrocylindrium sp.* [51], *Asp. awamori* [52], *Asp. niger A* and *B* [53, 54], *Asp. saitoi* [55], *Asp. terricola* [56], *Cladosporium sp.* [57], *Paecylomices var.* [51], *Pen. duponti* [58], *Pen. janthinellum* [59], *Mucor pusillus* [55], *Mucor miehei* [60], *Rhizopus chinensis* [55, 61], *Rhodotorula glutinis* [62, 64], *Pen. roqueforti* [64], and *Trametes sanguinea* [51].

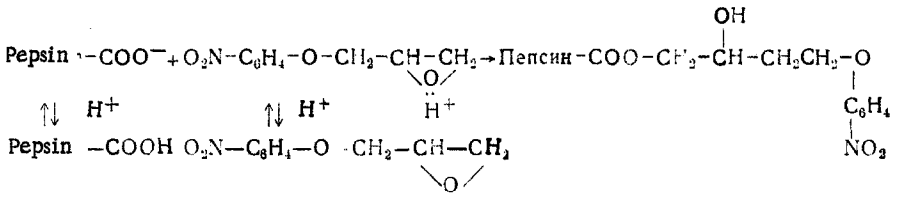
It must be mentioned that the pH dependence and rate of the reaction of other proteinases may be different from those of porcine pepsin. Thus, porcine pepsin C [42] is inactivated by DAN at pH 4.5, which is substantially lower than the pH of the inhibition of the pepsin A (pH 5.6). In the reaction of chymosin with the same inhibitor, a well-defined maximum of the inactivation is observed at pH 5.6. A change in the pH substantially retards the reaction. At pH 4.7, N-diazoacetyl-N'-DNP-ethylenediamine scarcely reacts with chymosin, but at pH 5.6 complete inactivation with the inclusion of one inhibitor residue is observed after 40 min [65]. At pH 5.0, chymosin does not react at all with 4-bromo-2-diazoacetophenone [56], which is probably connected with an unsuitable choice of conditions for the inhibition reaction.

For a number of carboxylic proteinases the sequence of amino acids round the reactive residue has been established with the aid of diazocarbonyl inhibitors and has shown a high degree of homology:

porcine pepsin A [33] — Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu
porcine pepsin C [42] — Ile-Val-Asp-Thr
bovine pepsin [40] — Ile-Val-Asp-Thr-Gly-Thr-Ser
chymosin [67] — Ile-Val-Asp-Thr-Gly-Thr, Ser-Leu
cathepsin D [68] — Ile-Ala-Asp/Thr, Gly, Thr, Ser/
aspergillopepsin A [54] — Ile-Ala-Asp
penicillopepsin [52] — Ile-Ala-Asp-Thr-Gly-Thr-Thr-Leu
proteinase from *Rhodotorula glutinis* [63] — Ile-Ala-Asp.

Reactions with Epoxides. Modification with epoxides has permitted the identification of a second catalytically active carboxy group in the carboxylic proteinases. Epoxy compounds are capable of esterifying carboxy groups in acidic solutions. The hydrolysis of epoxides to the corresponding glycols catalyzed by hydrogen ions takes place fairly slowly in solutions with pH values above 2.5 and the esterification reaction predominates. Tang [69] has investigated a number of epoxy compounds in the reaction with pepsin: propylene oxide, 1,2-epoxybutane, 1,2-epoxy-3-phenoxypropane, and 1,2-epoxy-3-p-nitrophenoxypropane (EPNP). The best inhibitor was EPNP. The UV spectrum of the protein modified by it had an additional maximum at 315 nm, which permitted the inclusion of the inhibitor to be determined. At pH 4.6 and 23°C, EPNP completely inactivated pepsin in 72 h, this process being accompanied by the inclusion of two inhibitor residues in the molecule. A change in pH from 2.2 to 5.6 did not affect the course of the reaction. Its specificity was increased by performing inhibition at 4°C [70]. After 116 h, the pepsin was 73% inactivated with the inclusion of 0.87 of an inhibitor residue, which indicated a modification of a functionally important group of the protein. The reaction scheme suggested by Hartsuck and Tang [70] assumes the participation

of a protonated form of the epoxide and an unprotonated carboxy group with an anomalously low pK_a value in the reaction:



From pepsin modified by two EPNP residues, Chen and Tang [71] isolated a number of peptides and showed that the epoxide alkylates Met-290 and esterifies Asp-32. In addition, they isolated a tripeptide the sequence of which coincided with the environment of Asp-215. As Stepanov et al. [72] have reported, it is obvious that, under the conditions of inhibition of pepsin, Asp-215 is involved in the reaction as well as Asp-32. Peptides that were analogs of pepsin substrates protected one of the groups from modification.

Reactions with EPNP similar to that of porcine pepsin take place with the pepsins of man [69], monkeys [39], and chickens [41], with gastricsin [69], with cathepsin [73], with the proteinase from seminal fluid [74], and with the proteinases of the mold fungi *Acrocylindrum sp.* [51], *Asp. awamori* [75], *Asp. niger A* [53], and *B* [51], *Asp. saitoi* [76], *Mucor pusillus* [77], *Paecylomyces var.* [51], *Scytalidium lignicolumn* [78], and *Trametes sanguinea* [76].

Tang [69] observed the inclusion of four residues of the inhibitor in chymosin, but Chang and Takahashi [43] observed the inclusion of only two residues, as in the case of porcine pepsin. One of them could be eliminated by treatment with a 1 M solution of hydroxylamine. The bond of the second residue was stable, which could be explained by the alkylation of a methionine residue. The performance of the reaction at 4°C was accompanied by the inactivation of the chymosin and the inclusion of one inhibitor residue [43]. From chymosin modified with two EPNP residues it was possible to isolate peptides for one of which an amino acid sequence corresponding to the environment of Asp-32 was established.

Penicillopepsin does not contain methionine residues and therefore its molecule includes one EPNP residue, apparently as the result of the modification of a carboxy group [79].

In a number of carboxylic proteinases, amino acid sequences have been observed which are homologous with that which reacts with EPNP in pepsin and chymosin:

human pepsin [80] — Val-Phe-Asp-Thr-Gly
porcine pepsin [6] — Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn
bovine pepsin [81] — Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn
chymosin [82] — Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn
equine pepsin [13] — Ile-Phe-Asp-Thr
penicillopepsin [79] — Asn-Phe-Asp-Thr-Cly-Ser-Ala-Asn
aspergillopepsin A [11] — Asn-Phe-Asp-Thr-Gly-Ser-Ala-Asn
Rhizopus pepsin [0*] — Asx-Phe-Asp-Thr-Gly-Ser-Ala-Asn.

The high degree of homology of the amino acid sequence, and also the general nature of the EPNP inhibition reaction shows that the carboxy group of an active center that is characteristic for all carboxylic proteinases is involved in the reaction. Preliminary treatment of porcine pepsin [69], chymosin [43], and a number of fungal proteinases [51] with a diazo inhibitor has no effect on the subsequent inclusion of the epoxide. However, for some proteinases the reactions with EPNP and with diazocarbonyl compounds are interdependent. Thus, chicken pepsin 94% inhibited and containing 3 EPNP residues includes only 0.26 of a DAN residue [41]. Likewise, penicillopepsin inactivated by DAN does not react with EPNP [79]. Mains and Hofmann [79] assumed that the modification of one of the carboxy groups reduces the reactivity of the other and thereby prevents reaction with the second inhibitor.

Carbodiimides. Matyash et al. [83] studied the reaction of porcine pepsin with the colored mono-DNP-hexamethylenediamine in the presence of N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide. The reaction was accompanied by the inclusion of one residue of the colored amine and several carbodiimide residues in the protein molecule, probably through the

*As in Russian original — Publisher.

formation of N-acylurea groups. The activity of the pepsin fell to 20%. The inclusion of 2-3 carbodiimide residues was not reflected in the activity of the enzyme. It was found that in spite of the inclusion of one amine residue the carboxy groups of the C-terminal alanine and of Asp-243 and Glu-298 took part in the reaction [84]. These authors consider that the reaction is preceded by the sorption of mono-DNP-hexamethylenediamine by a hydrophobic zone on the surface of the pepsin. The sorbed amine can then react with any of the three carboxy groups activated by carbodiimide and spatially close to the hydrophobic zone. The formation of a covalent bond between the amine and a carboxy group fixes the DNP-amine in the binding zone and makes it inaccessible for other amine molecules. The Michaelis constants of the native and modified proteins for the hydrolysis of diphenyl sulfite coincide, but catalytic constant for the modified protein is 40% lower than for the native pepsin. On the basis of the results obtained, the authors drew the conclusion that modification affects not the binding of the substrate but the catalytic stage. Consequently, even one of the modifiable groups is important for the manifestation of catalytic activity by pepsin.

Cheeseman [66] has shown that the treatment of chymosin with a 750-fold excess of the same carbodiimide leads to the complete inactivation of the enzyme both in the absence and in the presence of the methyl ester of glycine. In the latter case, however, the glycine content of the protein is not increased. Keeping the modified chymosin in hydroxylamine solution restores the activity of the enzyme, which leads to the conclusion of the participation of tyrosine residues in the reaction.

The water-soluble colored N-(γ-dimethylaminopropyl)-N'-(p-phenylazophenylene)carbodiimide [85] and dicyclohexylcarbodiimide [86] react with pepsin to form N-acylureas, but with the inclusion of 3-6 carbodiimide residues the enzyme retains some activity. The water-soluble colored carbodiimide proved to be a selective reagent for aspergillopepsin A and the proteinases from *Asp. foetidus* and *Asp. niger B*, which, unlike pepsin, are complete inactivated by even a twofold excess of the reagent, with the inclusion of one carbodiimide residue [87]. It has been shown for aspergillopepsin A that the carboxy group of Asp-215 from the active center and also a carboxy group from the N-terminal part of the molecule, probably Asp-11, are involved in the reaction [88]. Dicyclohexylcarbodiimide also inactivates aspergillopepsin A with the inclusion of one carbodiimide residue, but a 21-fold excess of the reagent is necessary for this [89].

Porcine pepsin reacts with trimethyloxonium tetrafluoroborate at pH 5.0 and 37°C [90]. Three carboxy groups are modified most rapidly but the pepsin retains 100% of its activity. Exhaustive modification leads to the inclusion of 11-13 methyl groups and to 97% inactivation. An indirect calculation permits the conclusion that among the slowly reacting groups there are at least two which are important for catalytic activity.

Nakagawa et al. [91] have used hydroxylamine as a "nucleophilic trap" for revealing catalytically important carboxy groups in pepsin that participate in the hydrolysis of sulfite esters. The reaction of pepsin with phenyl tetrahydrofuryl sulfite was carried out in hydroxylamine solution at pH 5.3. At a molar ratio of ester and pepsin of 2.5:1, one mole of hydroxamate was formed and the activity fell by 20%. An increase in the concentration of substrate and a lengthening of the time of the reaction were accompanied by the inclusion of 3-4 hydroxamate residues in the protein molecule and 50% inactivation. In a pepsin hydrolysate of the enzyme containing 3.3 molecules of hydroxamate three hydroxamate-containing peptides were found. One of them corresponded in its composition to the Asp-32 fragment and another to the Asp-215 fragment. The location of the third peptide could not be determined. These authors consider that the detection of hydroxamate is a direct proof of the formation of intermediate mixed anhydrides in the hydrolysis of sulfite esters by pepsin. The fact that Asp-32 and Asp-215 are involved in the reaction with hydroxylamine confirms that hypothesis that one and the same active center is responsible for both the proteinase and the sulfite esterase activities. However, the inclusion of 3-4 molecules of hydroxamate shows that the formation of anhydrides for several carboxy groups is possible. The participation of other carboxy groups in the hydrolysis of sulfide esters can explain the retention of 100% activity with respect to the substrate of a pepsin modified with EPNP or p-bromo-α-diazoacetophenone [92].

Modification of Amino Groups. Under the action of ketene [93], pepsin loses all its free amino groups but retains its activity completely. Under the deaminating action of nitrous acid [94], the enzyme retains 60% of its activity. Partial inactivation may be connected with the modification of two tryptophan residues. The treatment of pepsin with ethoxy-

formic anhydride is accompanied by the inclusion of one residue of the reagent and affects the N-terminal isoleucine, with the complete retention of activity [95]. The modification of the single lysine residue in porcine pepsin by the S-carboxymethyl mercaptide of p-mercuribenzoic acid [96] and by pyridoxal phosphate [97] does not affect the enzymatic activity. A comparison of the C-terminal regions of porcine pepsin [6], bovine pepsin [98], chymosin [99], and pencillopepsin [8] shows that the lysine residue is not invariant, and human pepsin and gastricsin [100], and also equine pepsins [13] do not contain any lysine at all. Consequently, the lysine residue can hardly play a functional role. However, the action of dimethylaminoaphthalenesulfonyl chloride on chymosin leads to 70% inactivation, accompanied by the inclusion of one residue of reagent in the protein molecule [101]. DNS-Lysine was found in the hydrolysis products. The authors assume that the lysine residue plays a fundamental role in the activity of chymosin.

Modification of Arginine. Porcine pepsin contains two arginine residues located in the C-terminal part of the molecule. In human pepsin [100], gastricsin [100], bovine pepsin [98], and chymosin [99], arginine residues are present in homologous sequences, which may show an important role of these residues. The choice of reagents that can be used for modifying the arginine residues in proteins is small, and only phenylglyoxal and butane-2,3-dione (biacetyl) are suitable for reaction in the pH range within which pepsin is stable. Kitson and Knowles [102] have found that pepsin loses 45% of its activity on treatment with phenolglyoxal at pH 5.6 and 20°C for 4 h. Only one arginine residue takes part in the reaction. The Michaelis constants of the modified and native proteins did not differ and, consequently, the modification did not affect the binding of the substrate. Reaction with biacetyl at pH 5.6 and 20°C for 24 h led to 85% inactivation of the enzyme and was accompanied by modification of the Arg-316 residue [103]. In the presence of acetyl-L-Tyr-L-Tyr inactivation was somewhat reduced with a parallel decrease in the degree of modification of the arginine. Human pepsin, gastricsin, and chymosin are inhibited by biacetyl in just the same way as porcine pepsin. Modification with biacetyl is not reflected on the capacity of pepsin for reacting with specific inhibitors — DAN and EPNP. Hartsuck and Tang [70] assume that an arginine residue participates in enzymatic catalysis, polarizing the carbonyl group of the bond being cleaved in the substrate. In the opinion of Kitson and Knowles [102], arginine can form a salt bond with a carboxy group of the active center of the enzyme and, thus, promote the stabilization of the carboxy group with a low pK_a value.

Penicillopepsin contains no arginine but it reacts with biacetyl in the same way as pepsin [1]. Hofmann assumes that the modification of one of the residues analogous to arginine in porcine pepsin leads to a change in the activity of penicillopepsin. To the Arg-316 residue of pepsin corresponds a serine residue in pepsin which cannot be modified with biacetyl. To Arg-308 corresponds the chemically similar lysine residue, and therefore Hofmann assumes that Arg-308 performs some function in the mechanism of the action of porcine pepsin. In actual fact, according to the results of x-ray structural analysis, in penicillopepsin Lys-308 forms a salt bond with Asp-304 and, thus, participates in the charge-transfer system [19].

Modification of Histidine. Human and porcine pepsins and gastricsin each contain one histidine residue. *Rhizopus* pepsin contains no histidine at all [1]. In porcine pepsin the histidine residue cannot be modified with ethoxyformic anhydride [95]. The photo-oxidation of chymosin [104] leads to complete inactivation and to 80% destruction of the histidine, which suggests an important role of histidine in the activity of the enzyme. The photo-oxidation of the proteinase from *Mucor pusillus* at pH 7.0 leads to 70% inactivation [105], the change in activity taking place in parallel with a decrease in the histidine content. There is little information on the chemical modification of histidine in carboxylic proteinases, but it is impossible to exclude a functional role for them in individual enzymes. According to James et al. [19], His-157 takes part in the charge-transfer system in penicillopepsin (in pepsin there is no analogous histidine residue).

Modification of Tyrosine. Treatment with ketene causes 40% inactivation of pepsin as the result of the acetylation of three tyrosine residues [106]. Acetylimidazole modifies 10-12 tyrosine residues [107]. The enzyme retains 10% of its activity with respect to hemoglobin, but the esterase activity rises to 180% and the peptidase activity increases by a factor of 2-2.5 [107, 108]. The modification of tyrosine apparently somewhat worsens the binding of large molecules but facilitates the binding of short peptides [109]. Chicken pepsin reacts with acetylimidazole in a similar manner to porcine pepsin [110]. The same effect is caused by the carbamoylation of porcine pepsin with potassium cyanate [111]. The action of a specific reagent — tetranitromethane — permits the finding of the number of "accessible"

tyrosine residues in the porcine pepsin molecule [112]. After 24 h, 4-5 of the 16 tyrosine residues have been modified, which is accompanied by a more than twofold decrease in the proteinase activity but with no change in the peptidase activity. Zavada et al. [113] found that the Tyr-9, Tyr-174, and Tyr-189 residues underwent nitration. The iodination of pepsin is accompanied by decreases in the proteinase, peptidase, and esterase activities [114, 115]. Treatment with a 13-fold excess of radioactive iodine led to 75% inactivation, 2.8 iodine atoms being incorporated in the protein molecule [116]. The Tyr-9 and Tyr-174 residues were affected with the incorporation of two iodine atoms. It is interesting that the adjacent Tyr-175 residue was not modified. The acid proteinases of *Pen. janthinellum, Endothia parasitica,* and *Rhizopus chinensis* are inactivated by iodine in just the same way as porcine penicillin. The inhibition of the milk-clotting enzyme from *Mucor pusillus* depends strongly on the pH. At pH 3-5 only 10% of the activity is lost, while at pH 6.7 the inhibition exceeds 90% [105]. *Rhizopus* pepsin is completely inactivated by p-benzenediazoniumtetrafluoroborate in 10 min [117]. Up to six tyrosine residues can be modified, but complete inactivation is observed with the modification of only three residues. The authors assume that at least one tyrosine residue is important for activity and is located in the active center of the proteinase. An investigation of the reaction of porcine pepsin with paranitrobenzenediazonium chloride over a wide pH range showed that loss of activity is already observed at pH 2.0 [113]. With a rise of the pH the rate of inactivation increases, and at pH 4.1 the enzyme is 90% inactivated in 75 h. The reaction involves the Tyr-189 residue which is apparently located in the substrate-binding section of the enzyme.

Modification of Tryptophan. The molecule of porcine pepsin contains five tryptophan residues. Treatment with N-bromosuccinimide for 2 min led to a fall in the activity of the pepsin to 10-15% [118]. Four of the five tryptophan residues take part in the reaction. N-Bromosuccinimide can react with methionine, and tyrosine and can make peptide bonds. A more selective reagent for the modification of tryptophan is 2-hydroxy-5-nitrobenzyl bromide. On modification with this reagent, the protein retains 70% of its activity. The Trp-141 and Trp-181 residues react [119], while Trp-39 and Trp-190 are inaccessible for the modifying agent in the active protein, and Trp-300 cannot be modified even after the reduction of the disulfide bonds and carboxymethylation. Chicken pepsin is 40% inactivated after treatment with 2-hydroxy-5-nitrobenzyldimethylsulfonium bromide [41]. The authors concerned consider that the reaction also affects other functional gropus — possibly histidine residues. Chymosin was also modified with this reagent, but the results obtained by different authors are contradictory. Hill and Laing [104] observed the modification of 50% of the tryptophan residues with no change in enzymatic activity, but according to Cheeseman [66] the inclusion of 1.5 inhibitor residues was accompanied by 40% inactivation.

Modification of Methionine. The treatment of pepsin with iodoacetamide or iodoacetic acid does not change the activity of the enzyme [120]. Hydrogen peroxide oxidizes 2.5 methionine residues out of the four in porcine pepsin and 1.6 residues out of the three in bovine pepsin [121]. The activity of the enzyme changes insignificantly. The oxidation of chymosin is likewise not reflected in the activity of the enzyme, although half the methionine residues are affected [104]. The reaction of porcine pepsin with EPNP at pH 4.6 and 25°C together with the esterification of the aspartic acid from the active center leads to the alkylation of Met-190 [70].

Erlanger et al. [122] have investigated the reaction of porcine pepsin with a number of α-haloketones: p-bromophenacyl bromide, p-chlorophenacyl bromide, α-bromo-α-phenylacetophenone, and 2-bromoindan-1,3-dione. At pH 3.5, p-bromophenacyl bromide caused 50% inactivation of the enzyme in 2.5 h and 78% inactivation in 24 h. The enzyme could be reactivated with mercaptoethanol. Gross and Morell [123] assumed that the carboxy group of an aspartic acid residue took place in the reaction. Recently, Tarasova et al. [124], making use of the colored 4-amino-α-bromo-3-nitroacetophenone, similar in structure to α-bromo ketones, have shown unambiguously that in modification a sulfonium salt with a methionine residue is formed. The inclusion of one inhibitor residue at pH 2.5 reduces the proteolytic activity by 45%. The Met-290 residue undergoes modification. In the spatial structure of pepsin and other carboxylic proteinases, Met-290 is located on the surface of the molecule close to the cleft of the active center. The addition of the inhibitor apparently closes off the access of the substrate to the active center. Human pepsin [37], monkey pepsin [39], the proteinase from seminal fluid [74], and proteinase from *Rhodotorula glutinis* [62] are inactivated by p-bromophenacyl bromide, like porcine pepsin. However, gastricsin [37], chymosin [43], chicken pepsin [41], cathepsin [45], penicillopepsin [59], *Rhizopus* pepsin [25], and the proteinases

from *Mucor miehei* [60] and *Penicillium duponti* [58] are not inhibited by this reagent. The presence of methionine in a definite section of the polypeptide chain permits an explanation of the previously not understood specificity of the reaction. The absence of inhibition is connected with the replacement of the methionine in position 290 by residues of other amino acids.

Modification of Cystine and Cysteine. Porcine pepsin does not contain free sulfhydryl groups. Nakagawa and Perlman [26] have studied the possibility of reducing the disulfide bonds in pepsin and pepsinogen. In the native protein, the cleavage of the Cys-202—Cys-210 and Cys-250—Cys-283 bonds takes place, which is accompanied by a decrease in the activity of the enzyme. The third disulfide bond, Cys-45—Cys-50, is reduced by mercaptoethanol only after denaturation of the protein in 8 M urea [127].

The proteolytic activity of chicken pepsin is close to that of porcine pepsin, but chicken pepsin scarcely cleaves small synthetic peptides which are good substrates for porcine pepsin. Chicken pepsin contains a free sulfhydryl group with the unusually low pK_a value of 7.5. The treatment of chicken pepsin with o-nitrophenylsulfenyl chloride is accompanied by the inclusion of one residue of the reagent [128] and an increase in the activity of the enzyme with respect to synthetic substrates with no change in proteolytic activity. Shechter et al. [129] have studied the action on chicken pepsin of a number of different compounds forming disulfide bonds or alkylating the sulfhydryl group of cysteine. In the native protein, the sulfhydryl group reacts only with aromatic or heterocyclic compounds and does not react with iodoacetamide, iodoacetate, and ethylenediamine. In all cases, chemical modification is accompanied by an increase in the rate of hydrolysis of low-molecular-weight substrates. The authors consider that the sulfhydryl group does not take a direct part in the catalytic act, but the cysteine residue is close to the substrate-binding zone. Modification of the sulfhydryl group facilitates the binding of small molecules and brings the structure of the active center of chicken pepsin closer to the structure of the active center of porcine pepsin.

In summarizing the facts relating to the chemical modification of carboxylic proteinases that have been considered, it must be observed that a change in activity may be connected with a modification of a whole series of functional groups that take part both in the catalytic act and in the binding of the substrate or the charge-transfer system. It may be hoped that the accumulation of facts on the tertiary structure and chemical modification of the enzymes will permit us to obtain an idea of the mechanism of the functioning of this class of enzymes.

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METHOD FOR DETERMINING THE POSITIONS OF SULFATE GROUPS IN SULFATED CARBOHYDRATES

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A new method of determining positions of the sulfate groups in sulfated carbohydrate-containing compounds is described which consists in the direct replacement of the sulfate groups by formyl groups through the action on these compounds of chloromethylenediethylammonium chloride in dimethyl formamide. Subsequent chromatomass spectrometry permits the determination of the positions of the formyl groups and, consequently, of the initial sulfate groups. The replacement of sulfate groups by formyl groups under these conditions takes place both in sulfated monomeric and polymeric compounds. The stability of the formyl group in peracetylated monoformates under the conditions of acetolysis has been shown, and in the case of polymeric compounds this permits the determination of the positions of formyl and sulfate groups.

Sulfated carbohydrate-containing compounds are widely distributed in nature and exhibit various biological activities [1-3]. The study of such compounds by the usual methods of carbohydrate chemistry is associated with certain difficulties, one of which is the determination of the positions of the sulfate groups in the molecule of a monosaccharide. The solution to this problem is important, since in a number of cases the biological activity is directly connected with the positions of the sulfate groups.

As a rule, in order to prove the positions of sulfate groups strictly it is necessary to use a combination of indirect methods permitting the localization of the sulfate groups to be judged fairly successfully in each concrete case.

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