Purification and Properties of Two Protease Inhibitors from Rat Skin Inhibiting Papain and Other SH-Proteases

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Two papain inhibitors, I_1 and I_2 , from rat skin extract were purified by affinity chromatography on KSCN-modified papain-agarose gel and by gel filtration on Sephadex G-100. I_1 had a molecular weight of 74 000, a pI of 4.6, and it contained 4 % of carbohydrates. I_1 inhibited papain, ficin, bromelain, rat skin benzoylarginine-2-naphthylamide hydrolase, and to a minor extent, rat skin cathepsin C and bovine trypsin. Bovine chymotrypsin or rat skin cathepsin D were not inhibited and benzoylarginine-2-naphthylamide hydrolase was inhibited only at alkaline pH. An inhibitor corresponding to I_1 was present in various rat tissues and also in serum. A similar inhibitor was present in the skin of cat, rabbit, guinea pig, and man. I_2 had a molecular weight of 13 400, a pI of 4.9 and it contained no carbohydrates. I_2 inhibited all thiol proteases tested, but not trypsin, chymotrypsin, or rat skin cathepsin D. I_2 formed an equimolar complex with papain and benzoylarginine-2-naphthylamide hydrolase. I_2 was present in rat skin, muscle, lung, and small intestine, but not in kidney, liver, or serum. A similar inhibitor was found in skin extracts of cat, rabbit, guinea pig, and man.

A number of inhibitors of serine proteases have been characterized from various plant and animal sources,¹ while only a few inhibitors of SH-proteases, such as papain (E.C. 3.4.22.2), cathepsin B1 (cathepsin B, E.C. 3.4.22.1), and cathepsin C (dipeptidyl peptidase, E.C. 3.4.14.1) are known. Finkensaedt² found that the supernatant fraction after differential centrifugation of rat liver homogenate inhibited cathepsins B and C, an observation not confirmed by Bouma and Gruber.³ A polysaccharide associated with mouse haptoglobin inhibits

bovine cathepsin B1, papain, and trypsin.^{4,5} However, human haptoglobin does not inhibit human cathepsin B1, while α_2 -macroglobulin is inhibitory.⁶ Chicken egg white contains an inhibitor with a molecular weight of 12 700 that inhibits the plant proteases papain and ficin (E.C. 3.4.22.3),^{7,8} and also the mammalian SH-proteases, cathepsins B1 and C.^{9,10} The inhibitor has different active sites for cathepsins B1 and C and it forms complexes with enzymatically inactive Hg^{2+} -derivatives of the enzymes.^{9,10} An inhibitor with properties similar to the egg white inhibitor has been demonstrated from Arthus lesions of rabbit^{11,12} and guinea pig¹³ skin and from rabbit skin burns.¹⁴ Sera of rabbit, guinea pig, and cow also contain an inhibitor that inhibits papain and a neutral inflammatory SH-protease.¹⁴ The serum inhibitor is contained in the α_1 -acid glycoprotein fraction and has a molecular weight of about 40 000–50 000.

During purification of rat skin benzoylarginine-2-naphthylamide hydrolase (BANA hydrolase), the presence of two inhibitors, I_1 and I_2 , of the enzyme in the skin was noticed,^{15–17} They also inhibited papain, and in this communication purification of the inhibitors, based on their papain inhibiting capacity, is presented. Some properties of the inhibitors are given and the presence of similar inhibitors in rat tissues and in skin of several mammalian species is demonstrated.

MATERIALS AND METHODS

Reagents. α -N-Benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA), α -D-methylmannoside, porcine chymotrypsinogen A, and

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equine cytochrome *c* were from Fluka AG. *N*-Acetyl-L-tyrosine ethylester (ATEE), casein (nach Hammarsten), ethylenediaminetetraacetic acid disodium salt (EDTA), and 4-dimethylaminobenzaldehyde were from E. Merck AG. Bovine hemoglobin (type II), bovine serum albumin, anthrone, indole, and Coomassie Brilliant Blue R-250 were from Sigma Chemical Co. Glycyl-L-arginine-2-naphthylamide (Gly-Arg-NA) was from Fox Chemical Co., dithiothreitol (DTT) from Calbiochem, and cyanogen bromide from Eastman-Kodak. Sepharose® 4B, Concanavalin A-Sepharose® 4B, Sephadex® G-100, Sephadex® G-200, and Blue Dextran were purchased from Pharmacia Fine Chemicals. Goat antiserum against rat serum proteins was obtained from Nordic Immunological Laboratories.

Enzyme preparations. Rat skin BANA hydrolase preparations AI and NII were obtained as described previously.¹⁶ Rat skin cathepsin D was purified as described by Heikkinen *et al.*¹⁸ A cathepsin C preparation was partially purified from rat skin extract by precipitation with acetone, gel filtration on Sephadex G-200, and chromatography on DEAE cellulose. The enzyme hydrolyzed Gly-Arg-NA at pH 5.0 producing glycylarginine and 2-naphthylamine as reaction products, and was activated by DTT. Papain (für biochemische Zwecke, E. Merck AG), ficin (crude, Sigma Chemical Co.), and bromelain (grade II, Sigma Chemical Co.) were dissolved in water (0.1 mg/ml) and used within one day. Bovine trypsin (type III, Sigma Chemical Co.) and chymotrypsin (type II, Sigma Chemical Co.) were dissolved in 1 mM HCl (0.1 mg/ml) and used within four days. Mercuripapain was prepared from Merck's papain by affinity chromatography on an organomercurial-agarose column according to Sluyterman and Wijdenes.¹⁹ The procedure increased the specific activity of papain 3.6-fold.

Inhibition assays. All assays were performed in duplicate. The incubation mixture was composed by pipetting 0.1 ml of an inhibitor solution, 0.1 ml of an enzyme solution, and 0.1 ml of a buffer to a test tube at room temperature. After 10 min, the tube was transferred to a 37 °C water bath, and 0.1 ml of a substrate was added. The mixture was incubated for 10 min, and the reaction products were assayed. The buffers were: 0.2 M Tris-HCl, pH 7.5, 4 mM DTT, 8 mM EDTA for papain, ficin, and bromelain; 0.2 M Tris-HCl, pH 8.0, 10 mM CaCl₂ for trypsin and chymotrypsin; Britton-Robinson buffer, pH 5.8,¹⁵ 0.8 mM DTT, 4 mM EDTA, 4 mM KCN for BANA hydrolase; 0.2 M sodium acetate buffer, pH 5.0, 0.2 M NaCl, 4 mM DTT, 4 mM EDTA for cathepsin C, and sodium lactate-acetate buffer, pH 4.3 for cathepsin D.¹⁵ BANA (5 mM) was used as a substrate for papain, ficin, bromelain, BANA hydrolase, and trypsin; Gly-Arg-NA (1 mM) for cathepsin C; hemoglobin (2 %) for cathepsin D, and ATEE (15 mM) or casein (1 %) for chymotryp-

sin. The reaction products were assayed as described previously.¹⁵⁻¹⁷ The inhibition was expressed as the amount of an enzyme (mg) inhibited by 1 ml of an inhibitor solution (U/ml) or by 1 mg of an inhibitor (U/mg). Linearities of the inhibition assays were always controlled by using serial dilutions of inhibitor solutions.

Other assay methods. Proteins were determined as described previously.¹⁵ Carbohydrates were assayed by indole and anthrone using glucose as a standard.²⁰

Tissue extracts. Long Evans rats weighing 150–200 g were used. Extracts of rat tissues and of rabbit, guinea pig, cat, and human (a female cadaver) skin were prepared as described previously,¹⁷ but the tissues were minced with scissors and homogenized in 10 mM sodium phosphate buffer, pH 6.0, 1 % KCl, using Ultra-Turrax TP-18 homogenizer. When large quantities of rat skin extract were needed, the skin was minced with a meat mincer and Ultra-Turrax T-45 homogenizer was used.¹⁵ The protein concentration of a typical rat skin extract was 2.1 ± 0.18 mg/ml and the specific papain inhibiting activity was 0.12 ± 0.4 U/mg.

Acetone precipitation. To 50 ml of extract 117 ml of cold (–18 °C) acetone was slowly added in a cooling bath (–10 °C), and the precipitated proteins were collected by centrifugation at 8000 *g*, at –10 °C, for 10 min. The sediment was dissolved in 5 ml of 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, and undissolved materials were removed by centrifugation at 8000 *g*, for 10 min, at +4 °C. The supernatant was filtered by suction through a Millipore® type AP prefilter and AA filter, and applied to a Sephadex G-100 column.

Affinity chromatography on papain-Sepharose. Sepharose 4B was activated by cyanogen bromide and washed according to March *et al.*²¹ The washed gel (100 ml) was suspended in 200 ml cold 0.2 M sodium bicarbonate, pH 9.5, and 1 g of papain, dissolved in 100 ml of the bicarbonate buffer, was added. The coupling and washing of the gel was performed as described by March *et al.*²¹ To 1 ml of the gel 6.1 mg of papain was coupled as estimated from the protein content of gel washings. The papain-Sepharose gel was suspended in water to obtain a total volume of 200 ml, and 194 g of KSCN was slowly dissolved in the suspension under continuous stirring (the final concentration of KSCN was 5 M). The pH of the suspension was adjusted to 4.0 by 2 M acetic acid, and the suspension was allowed to stand for 2 h at room temperature. This procedure destroyed the BANA hydrolyzing activity of the papain-Sepharose gel. The gel was washed in a glass funnel with 1.0 l of water, 0.5 l of 3 M KSCN in 20 mM trisodium phosphate, and 1.0 l of 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, and stored as a suspension in 10 mM phosphate buffer, 0.1 M NaCl, 0.02 % NaN₃, at pH 6.0. A column of 2.5 × 20 cm was packed with the inactivated papain-Sepharose

and equilibrated with 10 mM sodium phosphate buffer, 0.1 M NaCl, pH 6.0. 450 ml of rat skin extract was applied to the column by pumping it two times through the column at a flow rate of 100 ml/h. Unadsorbed material was eluted with 100 ml of the equilibrating buffer, collecting fractions of 10 ml. Nonspecifically adsorbed proteins were eluted with 100 ml of 3 M KCl in the buffer, and KCl was washed out by 100 ml of the equilibrating buffer. Elution of the inhibitors was performed with 200 ml of 20 mM trisodium phosphate, 0.1 M NaCl (pH 12.1). Finally, the column was washed with 100 ml of 3 M KSCN in 20 mM sodium acetate buffer, pH 4.0, and equilibrated with the starting buffer for a subsequent chromatographic run. The pooled inhibitors from papain-Sepharose affinity chromatography were concentrated to 40 ml on Diaflo® UM-10 (Amicon NV) membrane and applied to a column of Sephadex G-100.

Gel filtration. A column of Sephadex G-100 with dimensions of 5 × 90 cm was used in the purification procedure. The column was equilibrated with 10 mM sodium phosphate buffer, pH 6.0, 0.1 M NaCl, at a flow rate of 60 ml/h, and fractions of 10 ml were collected. A Sephadex G-100 column with dimensions of 2.6 × 32 cm was used to demonstrate inhibitor activities in various tissues and to estimate the molecular weights of purified inhibitors. The column was equilibrated with 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. A sample of 4 ml was applied to the column at a flow rate of 20 ml/h and fractions of 2 ml were collected. The column was calibrated by using Blue Dextran, bovine serum albumin, chymotrypsinogen A, and cytochrome c as standards, and the molecular weights of the inhibitors were estimated according to Andrews.²²

Chromatography on Concanavalin A-Sepharose. A column of Concanavalin A-Sepharose® 4 B with dimensions of 1.6 × 6 cm was equilibrated with 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl, and 10 ml of a purified inhibitor preparation was applied to the column at a flow rate of 40 ml/h, collecting fractions of 3 ml. Unadsorbed proteins were eluted with 50 ml of the equilibrating buffer, and adsorbed glycoproteins²³ with 200 ml of 50 mM α -methyl mannoside in the buffer.

Isoelectric focusing. Preparative isoelectric focusings using Ampholine ranges 3.5–10 and 3–5 were performed as described earlier.¹⁶ Analytical isoelectric focusings were performed according to Wrigley.²⁴ Dialyzed samples containing 10 μ g of protein were entrapped in the gels during photopolymerization. After focusing, the gels were stained for proteins by Coomassie Brilliant Blue R-250²⁵ and for polysaccharides by the PAS reaction.²⁶ To determine the inhibitor activities of the focused bands, unstained gels were sliced longitudinally into halves. One was stained as described above, and the other cut transversely into 1.5 mm

pieces. The pieces were extracted for 20 h with 200 μ l of water, at 4 °C, and the papain inhibiting activities of the extracts were assayed as usual, but a dilute papain solution (0.02 mg/ml) and a long reaction time (60 min) was used.

Preparation of antisera. 0.5 ml of a purified inhibitor solution, containing 0.05 mg of protein, was emulsified with 0.5 ml of complete Freund's adjuvant and injected intradermally to the dorsal skin of a rabbit in ten aliquots. The injection was repeated three times at three weeks intervals using incomplete Freund's adjuvant. Eight days after the last injection blood was collected and serum prepared. The double diffusion method of Ouchterlony and immunoelectrophoresis on agarose plates, buffered with diethyl barbiturate buffer, pH 8.6, were used to test antigen-antibody reactions.

RESULTS

Evaluation of papain-Sepharose affinity chromatography. The rat skin inhibitors of BANA hydrolase also inhibited papain and were adsorbed on papain-Sepharose in a wide pH-range (pH 3–8). The adsorbed inhibitors could not be eluted by 1 mM HgCl₂, or by increasing or lowering the pH of the eluting buffer (pH 2–12), in the presence of 3 M KCl or 6 M urea. The inhibitors were, however, dissociated from papain-Sepharose by 3 M KSCN in 20 mM acetate buffer, pH 4.0. Repeated elutions with 3 M KSCN, at pH 4.0, destroyed the BANA hydrolyzing activity of the papain-Sepharose adsorbent. It was found

Fig. 1. Papain-Sepharose affinity chromatography. Eluents: 1: 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. 2: 10 mM phosphate buffer, pH 6.0, 3 M KCl. 3: 10 mM phosphate buffer, pH 6.0, 0.1 M NaCl. 4: 20 mM Na₂PO₄, 0.1 M NaCl. 5: 20 mM Acetate buffer, pH 4.0, 3 M KSCN. Other details are given in "Materials and Methods".

Fig. 2. Sephadex G-100 chromatography of the inhibitors from the papain-Sepharose affinity chromatography. ● papain inhibition. Δ BANA hydrolase inhibition. Details are given in "Materials and Methods".

that the inhibitors were adsorbed to a KSCN-inactivated papain-Sepharose, and that from the inactivated adsorbent they could also be eluted without KSCN by increasing the pH of the elution buffer to 8.9, or higher. The milder elution of the inhibitors from the inactivated adsorbent at alkaline pH resulted in an increased recovery of the inhibitors. Thus the papain-Sepharose gel was inactivated before use and the inhibitors were eluted at alkaline pH, as described in "Materials and Methods".

Purification of inhibitors. The papain-Sepharose affinity chromatography is depicted in Fig. 1. The inhibitors eluted with the alkaline buffer were pooled (fractions Nos. 56–65). The pooled inhibitors had a specific papain inhibiting activity of 1.23 U/mg. The yield was 42 % and the purification factor 11, as compared to the activity of the extract.

Concentrated inhibitors from papain-Sepharose affinity chromatography were sepa-

Fig. 3. Analytical isoelectric focusings of I_1 and I_2 . The pH range was from 3.5 (left) to 10.0 (right). The curves represent the effects of the focused inhibitors on papain activity (cf. "Materials and Methods"). a: PAS staining of I_1 . b: Coomassie Brilliant Blue staining of I_1 . c: Coomassie Brilliant Blue staining of I_2 . PAS staining of I_2 was negative and is not shown.

rated into two fractions I_1 and I_2 on Sephadex G-100 (Fig. 2). Pooled I_1 (fractions Nos. 71–82) had a specific papain inhibiting activity of 2.6 U/mg and pooled I_2 (fractions Nos. 120–132) 5.8 U/mg. The purification factors, as compared to the activity of the extract, were 24 for I_1 and 53 for I_2 . The total inhibitor yield ($I_1 + I_2$) was 27 % of the activity in the extract. A typical purification procedure is summarized in Table 1.

Properties of the inhibitors. The approximate molecular weights of 74 000 and 13 400 were obtained for I_1 and I_2 respectively.

Table 1. Summary of a typical purification procedure of the rat skin papain inhibitors.

Purification step	Volume ml	Protein mg/ml	Activity U/ml	Total activity U	Specific activity U/mg	Yield %	Purification factor
Extract	450	2.0	0.22	99	0.11	100	1
Papain-Sepharose	90	0.38	0.47	42	1.2	42	11
Sephadex G-100							
I_1	116	0.046	0.12	14	2.6	14	24
I_2	130	0.017	0.099	13	5.8	13	53

The carbohydrate content of I_1 was 4 %, while I_2 did not contain carbohydrates. As expected for a glycoprotein, I_1 was adsorbed to a column of concanavalin A-Sepharose and could be eluted with α -methyl mannoside in the buffer. The procedure did not increase specific activity of I_1 . I_2 was not adsorbed to concanavalin A-Sepharose.

The isoelectric point of I_1 was 4.6 and that of I_2 4.9, as measured after preparative isoelectric focusing. Analytical isoelectric focusing of I_1 separated 4–5 protein bands, which were also stained by the PAS reaction for carbohydrates (Fig. 3). The papain inhibiting activity of I_1 was focused in two peaks, which coincided with the two main protein bands of I_1 . I_2 was focused in one major band and two minor bands, which were stained by the protein dye but not by the PAS reaction. The inhibitor activity of I_2 coincided with the main protein band.

At -18°C the inhibitors were stable at least 6 months, and at $+4^\circ\text{C}$, in the presence of NaN_3 (0.2 g/l), at least 30 days. I_2 retained all its inhibitor activity after an incubation period of 20 min in a boiling water bath. I_1 lost 2 % of its activity at 65°C , 32 % at 70°C , and 78 % at 75°C , during on incubation period of 20 min. The stability of the inhibitors was similar in 10 mM acetate buffer, pH 4.0, in 10 mM phosphate buffer, pH 6.0, and in 10 mM Tris buffer, pH 8.0, in the presence of 0.1 M NaCl.

Antiserum raised against I_1 was polyvalent. After immunoelectrophoresis anti- I_1 serum precipitated 6 bands from rat serum, although only one band was formed with I_1 . The precipitate of I_1 and the major band of serum were located in the α_1 -globulin fraction. In immunodiffusion according to Ouchterlony the precipitation band of I_1 fused with a heavy band of serum, which was a sum of several precipitates. Anti- I_1 serum did not form precipitates with I_2 .

The antiserum raised against I_2 formed a sharp precipitation band with I_2 and in addition, faint bands with I_1 and rat serum. After immunoelectrophoresis the precipitate of I_2 was located in the α_2 -fraction, while the precipitates of I_1 and serum were found in the α_1 -fraction. The results suggest that rat serum may contain a protein antigenically

Fig. 4. Inhibition of several proteases by I_1 and I_2 . 1: papain (10 μg). 2: mercuripapain (1.8 μg protein). 3: bromelain (10 μg). 4: ficin (10 μg). 5: trypsin (10 μg). 6: BANA hydrolase (1.2 μg). 7: cathepsin C (1.2 μg). 100 μl of I_1 solution contained 9 μg and I_2 solution 1.7 μg of protein.

Table 2. Inhibition of several proteolytic enzymes by I_1 and I_2 as calculated from the data in Fig. 4.

Enzyme	Inhibition (U/mg)	
	I_1	I_2
Papain	2.2	6.4
Mercuripapain	0.64	1.7
Ficin	2.5	10.6
Bromelain	2.2	8.4
BANA hydrolase	< 0.05	1.9
Cathepsin C	< 0.05	0.5
Cathepsin D ^a	0	0
Trypsin	0.1	0
Chymotrypsin ^a	0	0

^a Not shown in Fig. 4.

and electrophoretically similar to I_1 , while I_2 is not present in serum.

Rat skin BANA hydrolase (preparation NII) and cathepsin C did not form precipitates with anti- I_1 and anti- I_2 serums, indicating that during purification of the enzymes they are dissociated and separated from I_1 and I_2 . A commercial goat antiserum against rat serum proteins did not precipitate I_1 or I_2 .

Fig. 5. Effect of pH on inhibition of papain and BANA hydrolase by I_1 and I_2 . $9 \mu\text{g}$ and $27 \mu\text{g}$ of I_1 was used in inhibition assays of papain and BANA hydrolase, respectively. $1.7 \mu\text{g}$ of I_2 , $10 \mu\text{g}$ of papain, and $1.2 \mu\text{g}$ of BANA hydrolase were used. The buffer was Britton-Robinson universal buffer,¹⁷ containing 4 mM dithiothreitol and 8 mM EDTA. ○ No inhibitor, ● with inhibitor.

Inhibition of several proteolytic enzymes by I_1 and I_2 is presented in Fig. 4 and Table 2. I_2 inhibited all SH-proteases tested, but not at all trypsin, chymotrypsin, or cathepsin D. I_1 inhibited strongly the plant SH-proteases papain, ficin, and bromelain, while only a slight inhibition of cathepsin C and BANA hydrolase (at pH 5.8) was noticed. In addition, slight inhibition of trypsin was noticed, while chymotrypsin or cathepsin D were not inhibited. Only mercuripapain and BANA hydrolase (preparation AI) were pure enough to permit the estimation of molar ratios of enzyme-inhibitor complexes. One mg of I_1 inhibited 0.64 mg of mercuripapain and one mg of I_2 1.7 mg of mercuripapain, or 1.9 mg of BANA hydrolase (the amount of mercuripapain was determined by the Lowry-method and is given as milligrams of protein). Using the molecular weights of the inhibitors and enzymes (23 000 for papain and 27 000 for BANA hydrolase) it can be calculated that one mol of I_1 inhibits 2.1 mol of mercuripapain, while one mol of I_2 inhibits

Fig. 6. a. Sephadex G-100 chromatograms of rat serum and acetone precipitated tissue extracts. b. Sephadex G-100 chromatograms of acetone precipitated tissue extracts.

1.0 mol of mercuripapain or 0.94 mol of BANA hydrolase. The results suggest the molar ratio of 2:1 for papain- I_1 complex and 1:1 for papain- I_2 and BANA hydrolase- I_2 complexes. The inhibitors did not hydrolyze BANA, Gly-Arg-NA, or hemoglobin.

Inhibition of papain by I_1 and I_2 and inhibition of BANA hydrolase by I_2 was only slightly affected by pH of the assay solution (Fig. 5). In contrast, inhibition of BANA hydrolase by I_1 increased 7-fold, when pH of the assay mixture was changed from 6 to 8.

Presence of inhibitors in tissues. In Fig. 6 a and b fractionation of papain inhibitors of

various rat tissues and of rabbit, guinea pig, cat, and human skin is presented. An inhibitor corresponding to the elution volume of I_1 (fraction No. 32) was present in rat serum and in all tissue extracts studied, and also in human serum (not shown in Fig. 6). An inhibitor corresponding to I_2 was present in all skin extracts studied and in rat lung, muscle, small intestine, and slightly in spleen, but was not found in rat serum, kidney, or liver.

DISCUSSION

The fact that the rat skin inhibitors of cathepsin B₁-like benzoylarginine-2-naphthylamide hydrolase also inhibited papain¹⁸ suggested that papain immobilized on agarose beads could be used as an affinity chromatography material in the purification of the inhibitors. Indeed, the inhibitors were bound to papain-Sepharose and the problem was how to elute them in active form. The inhibitors were successfully eluted with 3 M KSCN, at pH 4.0. SCN⁻ is known to be a chaotropic ion that causes dissociation of multienzyme complexes,²⁷ antigen-antibody complexes,²⁸ and collagenase- α_2 -macroglobulin complexes.^{29,30}

The inhibitors could not be eluted from papain-Sepharose with Hg²⁺-ions, 3 M KCl, or 6 M urea, at pH 2–12 suggesting that free SH-group of papain, ionic forces, or hydrogen bonds are of minor importance for binding of the inhibitors. Similar results have been obtained by Sen and Whitaker,⁸ who failed to purify the egg white papain-ficin inhibitor on a ficin-CM-cellulose adsorbent.

Irreversible inactivation of papain-Sepharose by 5 M KSCN, at pH 4.0 weakened complexes of the inhibitors with the adsorbent, and after inactivation of the gel, the inhibitors were dissociated at alkaline pH, but not by 3 M KCl, at pH 6. The changes in Sepharose-bound papain caused by KSCN are not known. It is possible that papain is modified by thiocarbamylation or by denaturation. Denaturation of Sepharose-bound papain may be restricted by the numerous covalent linkages of papain with Sepharose in such a manner that inhibitors binding capacity of the gel is retained. Binding of protease inhibitors to inactive enzymes is known to occur in several cases. The egg white papain inhibitor forms complexes with inactive

papain and cathepsin B₁,⁷⁻⁹ and enzymatically inactive anhydrotrypsin, anhydrochymotrypsin and methyl-chymotrypsin form complexes with protease inhibitors.^{31,32}

KSCN-inactivated papain-Sepharose did not adsorb all papain inhibitors from rat skin extract. A possible reason is the presence of competing enzymes in the extract. Inactive proteins are also adsorbed to the papain-Sepharose column. This is understandable, because papain is known as a potent proteolytic enzyme that has an affinity for numerous proteins. Most, but not all of the inactive proteins that are adsorbed to papain-Sepharose, are eluted with 3 M KCl in the equilibrating buffer.

I_1 inhibited effectively plant SH-proteases, while a high excess of I_1 was needed to inhibit rat skin cathepsin C and BANA hydrolase. BANA hydrolase was more effectively inhibited at alkaline than at neutral or acidic pH, which may explain the presence of a high-molecular-weight BANA hydrolyzing compound in gel chromatograms of rat skin extract, when elution was performed at pH 8.0.¹⁸ At pH 7.5 such a high-molecular-weight aggregate was not noticed.¹⁶

An inhibitor corresponding to I_1 seems to be quite common in rat tissues and in skin of various species. The similar molecular size and immunological reactivity of I_1 and the rat serum papain inhibitor suggest that I_1 in rat tissues might be derived from serum. I_1 is an acid glycoprotein and may be similar to the papain and neutral SH-protease inhibiting substance isolated by Tokaji from sera of cow, rabbit, and guinea pig.¹⁴ Trypsin is also slightly inhibited by I_1 , but it is not known, whether this inhibition is caused by I_1 itself, or by a contaminating rat serum trypsin inhibitor.³³ Mouse haptoglobin is associated with a polysaccharide that inhibits papain, cathepsin B₁, and also trypsin.⁵ It is not known, if I_1 is related to the haptoglobin-polysaccharide complex.

I_2 inhibited all SH-proteases tested, of both plant and animal origin. An inhibitor corresponding to I_2 was present in several rat tissues and abundantly in skin of various species. Its absence from rat serum suggests its intracellular nature.

I_2 seems to be similar to the inhibitor purified from rabbit skin with healing Arthus inflamma-

tion and from rabbit skin burns.¹⁴ I_2 and the Arthus inhibitor^{11,12} have much in common with the chicken egg white papain inhibitor:⁷⁻¹⁰ they all inhibit thiol proteases, but not serine proteases, they have a molecular weight of about 13 000, contain no carbohydrates, and are resistant to high temperatures. In addition, both I_2 and the egg white inhibitor form equimolar complexes with papain, and also form complexes with enzymatically inactive papain.⁷⁻⁸ It is not known if I_2 is, like the egg white inhibitor, a multihead inhibitor with different active sites for cathepsin B1 (or papain) and cathepsin C.¹⁰

The specific activity of a rat skin BANA hydrolase preparation NII, which had been purified 250-fold, increases markedly when the enzyme is highly diluted, or preincubated at pH 4, at 55 °C, for 20 min, before enzyme assay.^{16,17} The presence of a dissociable inhibitor in the enzyme preparation has been suggested.¹⁷ The antisera raised against purified rat skin inhibitors I_1 and I_2 did not form immunoprecipitates with BANA hydrolase preparation NII, suggesting that the effect of dilution on BANA hydrolase activity is not caused by I_1 or I_2 .

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