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STUDIES ON THE ELASTASE–SERUM PROTEIN INTERACTION

III. THE ELASTASE INHIBITORS OF SWINE SERUM WITH EMPHASIS ON THE ELASTASE–α_2-MACROGLOBULIN INTERACTION

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SUMMARY

Impregnation of agar gel electrophoretograms of swine serum with elastase (pancreatopeptidase E, EC 3.4.4.7) followed by overlaying with elastin particles in agar revealed that swine serum contains three protein inhibitors of elastase, an α_0/ϱ_1-globulin, an α_2-globulin and a β_1-globulin. The α_0/ϱ_1- and α_2-globulins are analogous to the α_1-antitrypsin and α_2-macroglobulin of human serum while the β_1- globulin, the principal elastase inhibitor of swine serum, has no apparent human serum counterpart. The molecular weights and electrophoretic mobilities of the α_0/ϱ_1-globulin, α_2-macroglobulin and the β_1-globulin were found, respectively, to be: 72 200, $-6.1 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$; 960 000 (ref. 15), $-4.1 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$; 83 600, $-3.2 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot s^{-1}$. 1 ml of swine serum was capable of binding 0.6 mg of swine elastase.

Swine α_2-macroglobulin was purified from swine whole serum by fractional precipitation with $(NH_4)_2SO_4$ followed by ultracentrifugation, Sephadex G-200 gel filtration and DEAE-cellulose column chromatography. An inhibition curve (elastase activity *vs* increasing amounts of α_2-macroglobulin) revealed that 1.13 mole of α_2-macroglobulin (mol. wt 960 000) was capable of binding 1.0 mole of elastase (mol. wt 25 000). The dissociation constant for the α_2-macroglobulin–elastase complex was $4.5 \cdot 10^{-9}$. pH-stat experiments with α_2-macroglobulin–elastase mixtures (molar ratio α_2-macroglobulin:elastase >1.0) revealed an appreciable cleavage of peptide bonds within 16 h. Peptide bond cleavage was also demonstrated by gel filtration of 48-h digests on Sephadex G-50 and G-200 and by peptide mapping.

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INTRODUCTION

Studies on the inhibition of elastase (pancreatopeptidase E, EC 3.4.4.7) by serum and by specific proteins isolated therefrom have been the subject of many communications during the preceding ten years[1–6]. The major impetus for these studies is centered around the belief that elastase and its inhibitor(s) in serum play a role in diseases which involve elastin-containing structures, notably the vasculature[7]. Due to the paucity of human pancreas preparations, these studies, for the most part, have been carried out with a heterologous system, *i.e.* elastase from swine pancreas and serum of human origin. It was concluded, therefore, that a thorough examination of a homologous system would not only provide information relative to possible interspecies variations, but would also provide data of greater validity from the standpoint of comparative biochemistry.

Human serum was previously shown to contain two inhibitors of swine elastase, α_2-macroglobulin and α_1-antitrypsin[6]. Swine serum, by contrast, was found to contain three inhibitors of the same enzyme, one of which was α_2-macroglobulin. The present study presents information on the interaction of swine elastase with swine whole serum and with purified swine α_2-macroglobulin.

Accounts of these studies have appeared in preliminary communications[8,9].

MATERIALS AND METHODS

Swine elastase was prepared by column chromatography on DEAE-cellulose as previously described[10,11]. Trypsin, from swine pancreas, was a 5 times crystallized product of the Armour Chemical Company and was kindly supplied by Dr W. R. Thomas. It was further purified by a single passage through DEAE-cellulose as previously described for elastase.

Swine serum was obtained from the Colorado Serum Company, Denver, Colorado.

Interaction of elastase and swine whole serum

Detection of elastase and trypsin inhibitors in swine whole serum. Elastase inhibitors were detected in swine serum by impregnation of agar gel electrophoretograms (of serum) with elastase followed by overlaying with elastin particles in agar. The method has been previously described in detail[6].

Trypsin inhibitors were detected in swine and human serum using an analogous method. Hemoglobin substrate powder (0.2 g; Worthington Biochemicals) was dissolved in 100 ml of 0.027 M diethylbarbiturate buffer (pH 8.6) and filtered (Whatman No. 1). The filtrate was brought to 100 ml with the above buffer, 1 g of agar was added and dissolved by heating (the hemoglobin was precipitated at this time as fine particulate material). Following zone electrophoresis in agar gel, the electrophoretograms were immersed for 40 min in a solution containing 20 μg/ml of trypsin (0.027 M buffer). After impregnation with trypsin, the agar plates were gently rinsed with a fine stream of distilled water and the hemoglobin–agar (70 °C) poured over the electrophoretograms as described for elastase[6]. Following incubation at room temperature overnight, the plates (elastin or hemoglobin–agar overlays) were immersed in 1% NaCl (24 h) for removal of serum protein and products of proteolysis. The plates were

either placed in distilled water for 1 h prior to drying and staining with Amido Black 10 B or were photographed directly using darkfield illumination (Fig. 1).

Determination of electrophoretic mobilities and molecular weights using swine whole serum. Sephadex G-200 (4 cm × 100 cm) and DEAE-cellulose (2.1 cm × 80 cm) columns were employed as previously described for the purpose of obtaining fractions rich in a single inhibitor. These fractions were then used for the determination of electrophoretic mobilities, relative to albumin, as previously described[6].

Every third Sephadex G-200 fraction (4 ml) exhibiting absorbance at 280 nm was lyophilized, dissolved in 0.5 ml of distilled water and 5 μl subjected to zone electrophoresis in agar gel. The gel layers were treated as above (Detection of elastase and trypsin inhibitors in swine serum) for the location of elastase inhibitors in the chromatogram. Location of the inhibitors within specific fraction numbers permitted the calculation of the apparent molecular weights. A procedure for the calculation of the molecular weights utilizing immunoelectrophoresis of Sephadex G-200 effluent fractions has been previously described[12].

The equivalence point of the reaction between elastase and swine whole serum was determined using hemoglobin as substrate, from an inhibition curve as previously described[6]. A dissociation constant for the elastase–inhibitor(s) complex(es) was calculated according to Green and Work[13] using 25 000 as the molecular weight of elastase.

Elastase–serum protein complexes were detected in agar gel electrophoretograms of elastase–serum mixtures as previously described[6].

Interaction of elastase and α_2-macroglobulin

Isolation of swine α_2-macroglobulin. α_2-Macroglobulin was isolated from swine serum as previously described for human serum[14]. Briefly, the method involves $(NH_4)_2SO_4$ fractionation, centrifugation in the Beckman Model L ultracentrifuge, Sephadex G-200 gel filtration, and finally two passages through a DEAE-cellulose column. Lyophilization was found to partially denature the protein and thus was avoided.

Equivalence point of the α_2-macroglobulin–elastase interaction and dissociation constant of the α_2-macroglobulin–elastase complex. These parameters were determined using graded amounts of purified α_2-macroglobulin and a constant amount of elastase in the presence of hemoglobin substrate. The procedure was carried out as described for whole serum.

Analysis of α_2-macroglobulin–elastase reaction mixtures. Previous experiments with human α_2-macroglobulin–elastase reaction mixtures showed that the inhibition of elastase was of the "temporary" type and that α_2-macroglobulin was slowly digested by the enzyme[6,14]. Therefore, in an effort to determine if such was also the case with swine α_2-macroglobulin, time-course α_2-macroglobulin–elastase mixtures were subjected to gel filtration, agar gel electrophoresis, peptide mapping and analysis in the pH-stat.

Gel filtration. Elastase (1 mg) was added to 51.5 mg α_2-macroglobulin. The molar ratio of α_2-macroglobulin (mol. wt 960 000, ref. 15) to elastase (mol. wt 25 000, ref. 16) was 1.34. The reaction mixture (in 6.0 ml 0.1 M NH_4HCO_3, pH 8.0) was incubated at 37 °C for 48 h and added to a 2.1 cm × 50 cm column of Sephadex G-50.

The breakthrough peak was pooled, concentrated to 5 ml with Sephadex G-200 and added to a 4 cm × 80 cm column of Sephadex G-200.

The extent of the digestion of α_2-macroglobulin by elastase was approximated by determining the area beneath the curves of the elution diagrams of the G-50 and G-200 column effluents. Areas of both breakthrough peaks were determined by making the trailing edge of each peak symmetrical to the leading edge. Therefore, the protein which comprised that portion of the peak(s) which was eliminated by the above extrapolation was assumed to possess a molecular weight(s) below the exclusion limit (Sephadex G-50, 10 000; Sephadex G-200; 200 000) of the gel(s). Following the last peak absorbing at 280 nm of the Sephadex G-50 chromatogram, 100 fractions comprising 600 ml of effluent were pooled, lyophilized, dissolved in 5 ml of 0.1 M acetic acid and added to a 2.1 cm × 50 cm column of Bio-Gel P 10 (Bio-Rad Laboratories), equilibrated with 0.1 M acetic acid. 1.0-ml volumes of the effluent fractions (5 ml) were analyzed by the ninhydrin method of Moore and Stein[17]. A single broad peak possessing several spikes was produced. Consequently the fractions comprising the entire peak were pooled, lyophilized and subjected to peptide mapping as described below.

Peptide mapping. This procedure was carried out as described by Katz *et al.*[18] using the devices recently described by this laboratory[19,20].

Samples (0.1 ml) of the 48-h digest described above were subjected to peptide mapping. Also, α_2-macroglobulin was oxidized with performic acid according to Hirs[21]. The oxidized protein (20.68 mg) was dissolved in 5 ml 0.1 M NH_4HCO_3 and incubated for 3 h at 37 °C in the presence of 1.0 mg of elastase. The elastase was added (1.0 mg/ml) in 0.5-ml aliquots at zero time and after 1.5 h had elapsed. The total digest was lyophilized, dissolved in 1.0 ml of distilled water and 0.2-ml aliquots subjected to peptide mapping.

The lyophilized material from the Bio-Gel P 10 run (see above) was dissolved in 1.0 ml of distilled water and 0.1-ml aliquots subjected to peptide mapping.

Interaction of α_2-macroglobulin and elastase at constant pH as followed in the pH-stat. pH-stat experiments were carried out essentially as described by Jacobsen *et al.*[22]. Radiometer equipment was used throughout. α_2-macroglobulin was thoroughly dialyzed against 0.1 M KCl and the protein concentration as determined by the method of Lowry *et al.*[23] was found to be 28.6 mg/ml. Elastase was used at a concentration of 2.5 mg/ml in 0.1 M KCl. 1.0 ml of α_2-macroglobulin solution was added to a 5 ml-reaction vessel, which was maintained at 37 °C and allowed to equilibrate for 10 min at that temperature under an atmosphere of CO_2-free nitrogen. Elastase (0.2 ml; 0.5 mg) was added and the reaction allowed to proceed at a maintained pH of 8.6 for 16 h. The molar ratio of α_2-macroglobulin:elastase was 1.49.

RESULTS

Interaction of elastase and swine whole serum

Electrophoretograms depicting the number and relative electrophoretic mobilities of elastase and trypsin inhibitors in swine serum are shown in Fig. 1. Following electrophoresis, the agar gel layers were impregnated with elastase and overlaid with elastin–agar (Fig. 1A) or hemoglobin–agar (Fig. 1B) or impregnated with trypsin and overlaid with hemoglobin–agar (Fig. 1C).

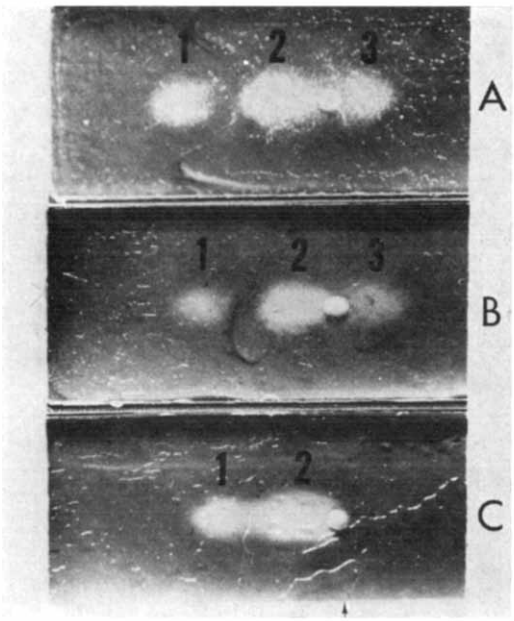

Fig. 1. Agar gel electrophoretic separation of the elastase and trypsin inhibitors of swine serum. (A) Following electrophoresis, the agar gel layers were impregnated with elastase, overlaid with elastin–agar, and incubated at room temperature (20 °C) overnight; 1, α_0/ϱ_1-globulin; 2, α_2-globulin; 3, β_1-globulin. (B) Impregnated with elastase and overlaid with hemoglobin–agar; other conditions and number designations as in A. 1% agar in diethylbarbiturate buffer, pH 8.6, I = 0.027; sample volume in all cases, 5 μl; 9.3 V/cm for 1 h; arrow indicates electrophoretic origins; all photographs by darkfield illumination. See text for details.

Fig. 1A shows that swine serum contains three inhibitors of elastase, a β_1-globulin, an α_2-globulin* and an α_0/ϱ_1-globulin**. When the serum electrophoretograms were impregnated with elastase and heat denatured hemoglobin used for substrate (hemoglobin–agar overlay) in place of elastin (Fig. 1B), results identical to those in Fig. 1A were obtained. Fig. 1C, however, shows that swine serum apparently contains only two inhibitors of trypsin as does human serum, an α_2-globulin (probably α_2-macroglobulin) and another protein which is apparently identical to the α_0/ϱ_1-globulin elastase inhibitor.

When samples of swine serum were subjected to immunoelectrophoresis and diffused against antiswine whole serum antibody, arcs of precipitate corresponding to the zones of undigested elastin (Fig. 1A) representative of the β_1-globulin and α_0/ϱ_1-globulin inhibitors were not apparent. In an effort to demonstrate precipitin lines for these proteins, the experiment presented in Fig. 1A was repeated, this time permitting electrophoresis to proceed for 2 h. These results are presented in Fig. 2. The arcs labeled 1 and 3 (Fig. 2B) are, in all probability, the proteins in question. These arcs were completely masked by albumin and transferrin in the usual 1-h runs.

It was previously shown that swine elastase–human serum mixtures when sub-

* Subsequent experiments showed this inhibitor to be α_2-macroglobulin.

** The electrophoretic mobility of this protein is more rapid than albumin, which has α_0 mobility, but slower than pre-albumin which has ϱ_1-mobility, hence the designation α_0/ϱ_1.

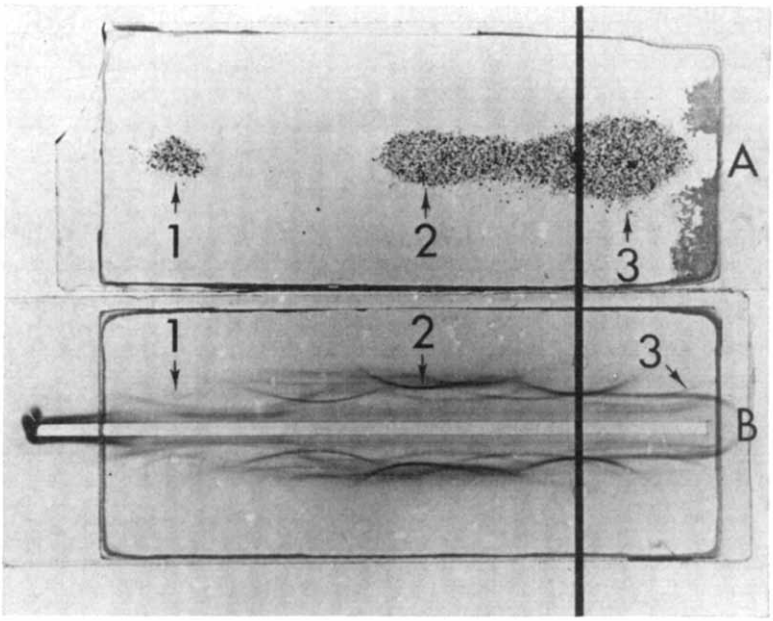

Fig. 2. Agar gel electrophoretic patterns of swine serum. (A) Impregnated with elastase and overlaid with elastin–agar. (B) Immunoelectrophoretogram of swine serum; developed with anti-swine whole serum antibody in trough. Numbers refer to elastin agar zones and arcs of precipitate probably representative of the inhibitors: 1, α_0/ϱ_1-globulin; 2, α_2-globulin; 3, β_1-globulin. 9.3 V/cm for 2 h; other conditions as in Fig. 1; vertical bar marks the electrophoretic origins. See text for details.

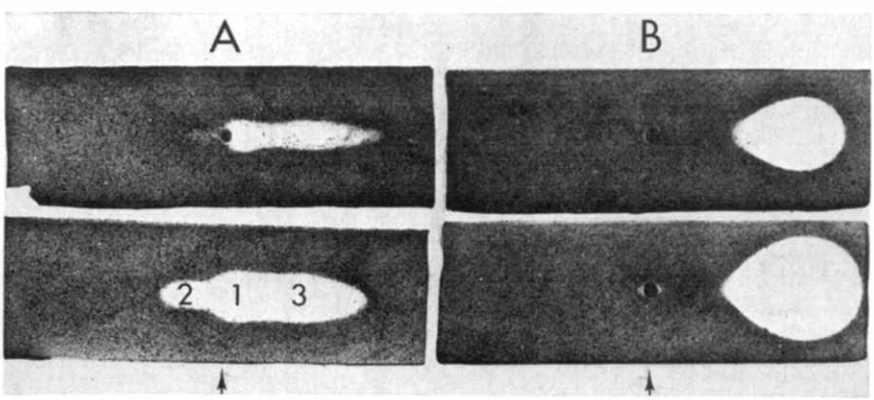

Fig. 3. Zone electrophoretic separation of elastase and elastase–swine serum mixtures and demonstration of elastase–inhibitor complexes. (A) Elastase–swine serum mixtures; 0.1 ml elastase (1.0 mg/ml) + 0.1 ml swine serum; top, incubated at 37 °C for 24 hours; bottom, incubated at 37 °C for 48 h. Numbers mark the positions of the elastase–serum protein complexes; 1, α_0/ϱ_1-globulin–elastase complex; 2, α_2-globulin–elastase complex; 3, β_1-globulin–elastase complex. (B) Elastase controls, 0.5 mg/ml; top, incubated at 37 °C for 24 h; bottom, 48 h at 37 °C. All slides overlaid with elastin–agar following electrophoresis. Other conditions as in Fig. 1. Arrows indicate electrophoretic origins. Anode is at the left.

jected to zone electrophoresis, in agar, overlaid following electrophoresis with elastin particles in agar, and incubated at 37 °C for 48 h, that elastase activity could be demonstrated at the positions of the complexes (α_2- and β_1-globulin positions)[6]. The results obtained with swine serum–elastase mixtures are presented in Fig. 3. It is readily apparent that free elastase is present at three distinct areas in the electrophoretograms. These areas of enzyme activity mark the positions of the dissociating complexes. Fig. 3A shows that the α_0/ϱ_1-globulin–elastase complex (designated 1; 48-h sample) migrates between the α_2-globulin–elastase (2) and β_1-globulin–elastase complexes. The results obtained for the α_2-globulin–elastase complex are identical to those obtained for the human serum α_2-macroglobulin–elastase complex[6]. The results for the α_0/ϱ_1-globulin–elastase complex are in close agreement with the results obtained for the human serum α_1-antitrypsin–elastase complex[6], *i.e.* the complex migrates as a β_1-globulin. The β_1-globulin–elastase complex migrates, electrophoretically, between the γ_1- and γ_2-globulin positions (Fig. 3).

The inhibition of elastase as a function of increasing amounts of swine serum, in the presence of hemoglobin, is shown in Fig. 4*. It is apparent that the inhibition of

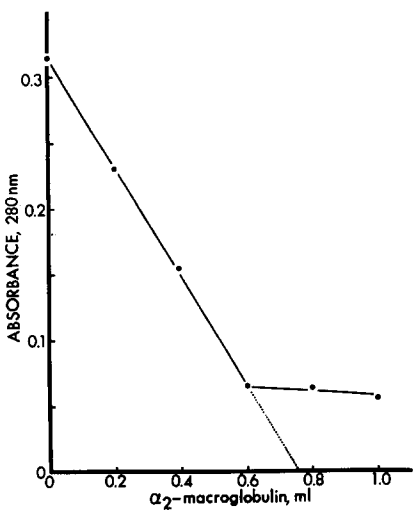

Fig. 4. Inhibition of elastase as a function of increasing amounts of swine serum at pH 8.6. Digestion mixture contained 2.0 ml of 2% hemoglobin substrate, 1 ml of elastase (50 μg), the indicated amount of serum and diethylbarbiturate buffer, I = 0.027, to a total volume of 4 ml; incubated at 37 °C for 30 min. Equivalence point is designated by dashed line from stoichiometric portion of the plot. See text for details.

elastase by swine serum is stoichiometric until the equivalence point is approached. These results parallel those obtained with human serum (Fig. 4)[6]. Extrapolation of the stoichiometric portion of the curve to the abscissa gave an equivalence point of 1 ml swine serum = 0.6 mg elastase (Fig. 4). A dissociation constant for the elastase–inhibitor complexes calculated from the inhibition curve (Fig. 4) according to Green and Work[13] was found to be $K = 2.5 \cdot 10^{-8}$.

* This extrapolation to 100% inhibition for the determination of the equivalence point is necessary due to the dissociation of the enzyme–inhibitor complex(es) and is justified on the basis of the stoichiometric nature of the reaction.

TABLE I

APPARENT MOLECULAR WEIGHTS AND ELECTROPHORETIC MOBILITIES OF THE ELASTASE INHIBITORS OF SWINE SERUM

See text for details.

Protein	*Apparent molecular weight*	*Electrophoretic mobility* $\times 10^5$ $(cm^2 \cdot V^{-1} \cdot s^{-1})$
α_2-Macroglobulin	960 000*	−4.1
β_1-Globulin	83 600	−3.2
α_0/ϱ_1-Globulin**	72 200	−6.1

* Armand and Guinand[15].

** The mobility of this protein is more rapid than albumin which has α_0 mobility but slower than pre-albumin which has ϱ_1 mobility, hence the designation α_0/ϱ_1.

The data presented in a previous publication[12] showed that it was possible to determine the apparent molecular weight of a protein by gel filtration on Sephadex G-200 in a mixture as complex as serum provided the void volume of the column (V_0), the elution positions (V) of two well-characterized proteins and the elution positions of the unknowns were known. With a knowledge of the latter parameters and the elution positions of the inhibitors, the molecular weights (exclusive of α_2-macroglobubulin) of the inhibitors were determined. These results appear in Table I. The results show a similarity in the molecular weight of human α_1-antitrypsin (3.5-S α_1-glycoprotein) and the α_0/ϱ_1-globulin of swine serum. The β_1-globulin elastase inhibitor has no apparent human serum counterpart.

Selected individual fractions from DEAE-cellulose chromatograms of swine whole serum which contained only one inhibitor were subjected to zone electrophoresis in agar impregnated with elastase and overlaid with elastin–agar. The resulting zones of undigested elastin were used in the determination of the electrophoretic mobilities of the inhibitors. These results are also presented in Table I.

Interaction of elastase and α_2-macroglobulin

An immunoelectrophoretogram of the α_2-macroglobulin used in these studies is

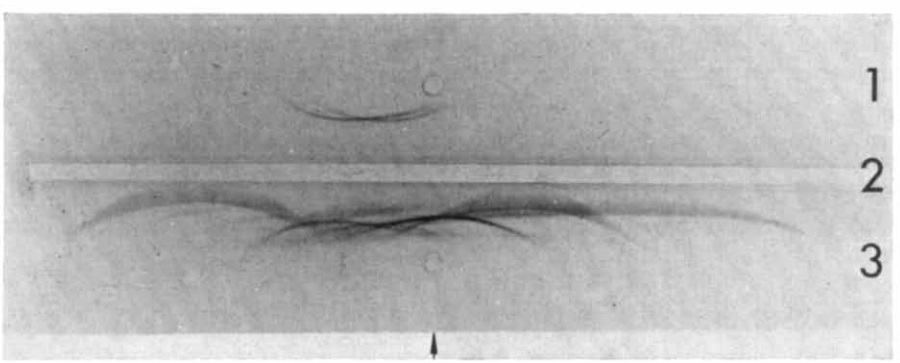

Fig. 5. Immunoelectrophoretic pattern of purified swine α_2-macroglobulin. α_2-Macroglobulin concentration, 5 mg/ml; 1, α_2-macroglobulin; 2, antiswine whole serum antibody; 3, swine serum. Sample volume, 1 μl; 1% agar in diethylbarbiturate buffer, pH 8.6, I = 0.027; 9.3 V/cm for 1 h; arrow indicates electrophoretic origin.

shown in Fig. 5. It is apparent that two arcs of precipitate are present. These results parallel those obtained by Armand and Guinand[15]. The product produced a single peak in the ultracentrifuge ($s_{20,w}$ 18.8) and single symmetrical peak when subjected to gel filtration on a 4 cm × 100 cm column of Sepharose 4B (Pharmacia). These results show that the minor contaminant possesses a molecular weight similar if not identical to α_2-macroglobulin and suggest that the purification procedure might have been the source of this protein. An arc of similar electrophoretic mobility can certainly be found upon critical examination of the whole serum pattern shown in Fig. 5. However, in a mixture as complex as serum one would expect this to be the case. Under any circumstances, the presence of the contaminant, (be it the result of the purification procedure or a *bona fide* protein of serum) in small concentration would not invalidate the data presented.

Equivalence point of the reaction between elastase and α_2-macroglobulin and the dissociation constant of the α_2-macroglobulin-elastase complex. The inhibition of elastase as a function of increasing amounts of α_2-macroglobulin in the presence of hemoglobin substrate is shown in Fig. 6*. Like the human α_2-macroglobulin–elastase interaction, the reaction is stoichiometric until equivalence is approached. Extrapolation of the straightline portion of the plot (Fig. 6) to the baseline gave an equivalence

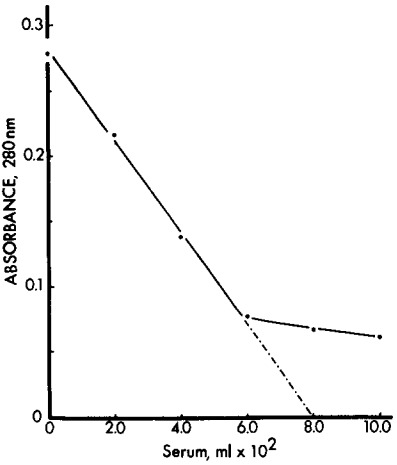

Fig. 6. Inhibition of elastase as a function of increasing amounts of swine α_2-macroglobulin. α_2-Macroglobulin concentration, 2.88 mg/ml. Elastase concentration and other conditions as in Fig. 4. See text for details.

point for the reaction of 2.17 mg swine α_2-macroglobulin = 0.05 mg elastase. Thus, 1.13 moles of α_2-macroglobulin (mol. wt 960 000, ref. 15) is capable of binding 1.0 moles of elastase. The dissociation constant of the α_2-macroglobulin–elastase complex calculated from this plot (Fig. 6) was $4.5 \cdot 10^{-9}$.

Agar gel electrophoresis of α_2-macroglobulin–elastase reaction mixtures. The 24-h and 48-h α_2-macroglobulin–elastase digests were subjected to agar gel electrophoresis followed by overlaying with elastin particles in agar and incubation for 24 h and 48 h

* See footnote on p. 187.

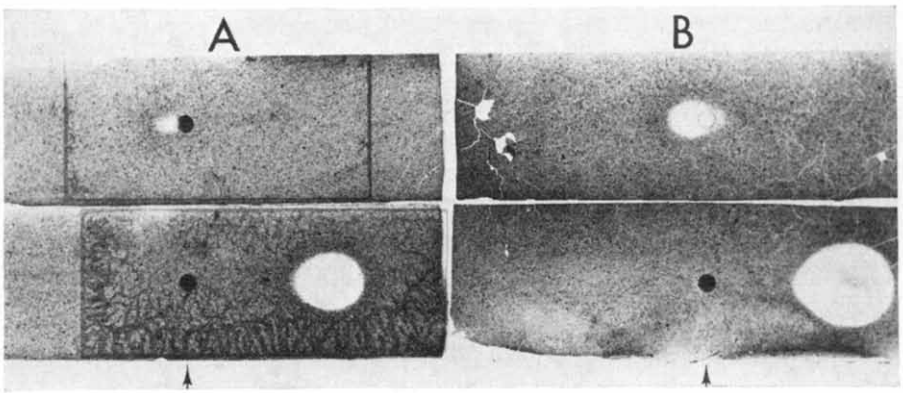

Fig. 7. Electrophoretic separation of α_2-macroglobulin–elastase digests. 51.5 mg α_2-macroglobulin + 1 mg elastase in 5 ml of 0.1 M NH_4HCO_3, pH 8.0. Total volume of reaction mixture, 5 ml. Incubated at 37 °C for 24 h (A) and 48 h (B). Sample volume 6 μl; other conditions as in Fig. 3.

at 37 °C. These results appear in Fig. 7. A slight amount of elastase activity can be demonstrated at the α_2-globulin position in the 24-h samples (Fig. 7A) with considerably more in the 48-h samples (Fig. 7B). The presence of free elastase at the position of the α_2-macroglobulin–elastase complex is evidence for either dissociation or degradation of the inhibitor.

Gel filtration of α_2-macroglobulin–elastase reaction mixtures. When a 48-h α_2-macroglobulin–elastase digest containing the equivalent of 51.5 mg of α_2-macroglobulin was added to a 2.1 cm × 50 cm column of Sephadex G-50, the profile shown in Fig. 8A was obtained. The area under the peaks (Table II) showed that approximately 80% of the digest contained proteins with molecular weights in excess of 10 000. Gel filtration of the breakthrough peak from the Sephadex G-50 run on Sephadex G-200 produced the profile shown in Fig. 8B. Therefore, 83% (Table II) of

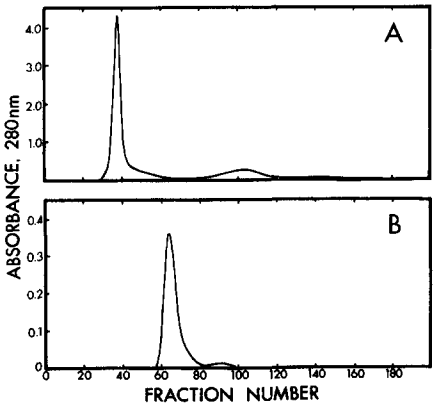

Fig. 8. Gel filtration of a 48-h α_2-macroglobulin–elastase digest. (A) Sephadex G-50, 2.1 cm × 50 cm. Sample volume, 6 ml (51.5 mg α_2-macroglobulin + 1 mg elastase in 6 ml 0.1 M NH_4HCO_3, pH 8.0); flow rate 10 ml/h; fraction volume, 5 ml; temperature was 4 °C; equilibrated with 0.1 M NH_4HCO_3, pH 8.0; temperature was 4 °C. (B) Sephadex G-200, 4 cm × 80 cm. Gel filtration of Fractions 30–42 from A. Flow rate 20 ml/h; other conditions as in A.

TABLE II

PEAK AREA ANALYSES OF SEPHADEX G-50 AND SEPHADEX G-200 CHROMATOGRAMS DERIVED FROM AN α_2-MACROGLOBULIN—ELASTASE DIGEST

See gel filtration profiles in Fig. 8 and text for details.

	Fractions	*Peak area (%)*
Sephadex G-50		
Peak 1	28–46	63.9
Peak 2	47–62	17.9
Peak 3	63–120	18.2
Sephadex G-200		
Peak 1	58–72	82.6
Peak 2	73–79	14.5
Peak 3	80–99	2.9

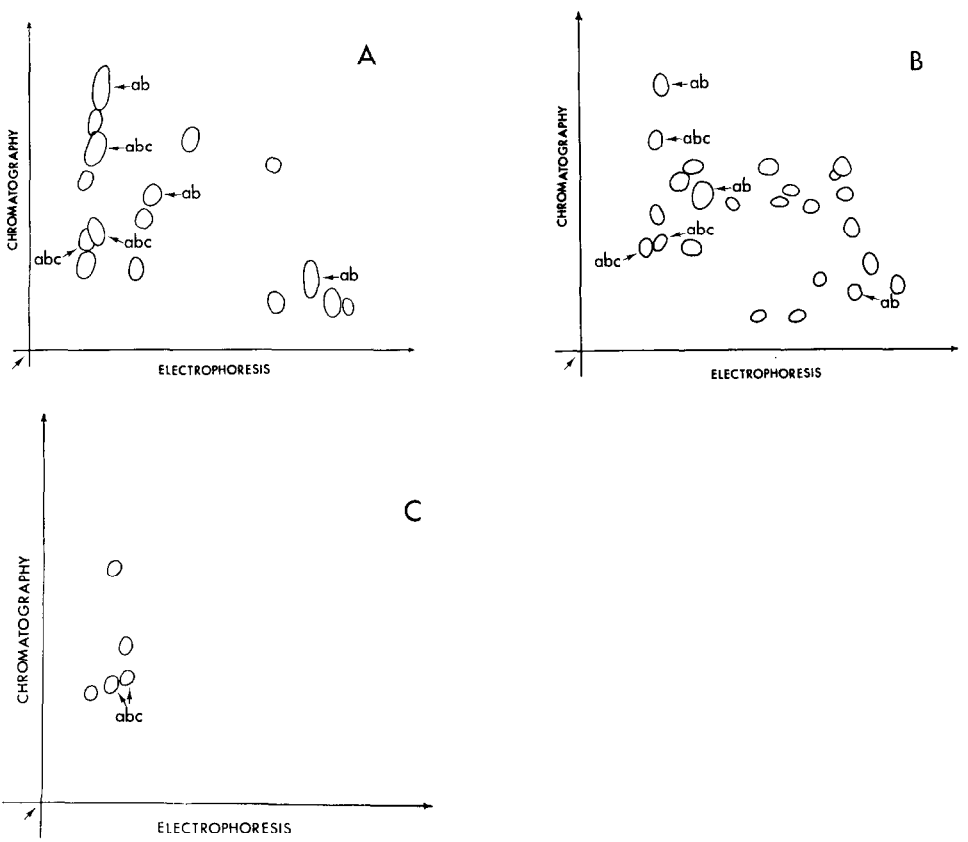

Fig. 9. Peptide maps of α_2-macroglobulin–elastase digests. (A) Peptide map of 48-h α_2-macroglobulin–elastase digest (0.1 ml) whose gel filtration profile is shown in Fig. 8A. (B) Peptide map of performic acid-oxidized α_2-macroglobulin–elastase digest. (C) Peptide map of 0.1 ml of concentrate of Fractions 120–240 from gel filtration profile in Fig. 8A. See text for details.

the protein present in Fractions 30–42 of the α_2-macroglobulin G-50 chromatogram possessed molecular weights greater than 200 000. The small area of 280 nm absorbing material in Fig. 8B and the trailing edge of the breakthrough peak probably represents α_2-macroglobulin fragments possessing molecular weights of less than 200 000.

Peptide mapping. The digestion of native α_2-macroglobulin by elastase is perhaps best exemplified by the peptide map of the 48-h digest shown in Fig. 9A. Fig. 9B is a peptide map of a 3-h digest of performic acid-oxidized α_2-macroglobulin and elastase. In Fig. 9C is presented a peptide map of Fractions 120–240 of the Sephadex G-50 column effluent. It is obvious that in the case of both the native (Fig. 9A) and the oxidized protein (Fig. 9B) an appreciable number of peptides were produced. This attests not only to the broad specificity of elastase but also to the fact that native α_2-macroglobulin is indeed digested by elastase.

For convenience, the peptides possessing similar or identical properties in the maps presented in Fig. 9 have been designated abc if present on all maps or ab if similar only on two. Three similar, if not identical, peptides were demonstrable in digests of native and oxidized α_2-macroglobulin (Fig. 9). Since the peptides shown in

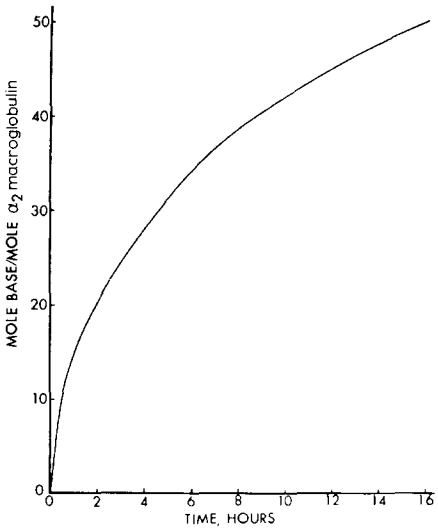

Fig. 10. pH-stat recording of the reaction between α_2-macroglobulin and elastase as a function of time. α_2-macroglobulin 28.6 mg + 0.5 mg elastase. Temperature and pH were maintained, respectively, at 37 °C and 8.6. The molar ratio of α_2-macroglobulin:elastase was 1.28. See text for details.

Fig. 9C were derived from the last 600 ml of the Sephadex G-50 column effluent, these compounds possess molecular weights of the order of 1000–2000 or less. Two of the four peptides were found to be present in the unfractionated digests (Figs 9A and 9B).

pH-stat recording of the digestion of α_2-macroglobulin by elastase. The results of this experiment are presented in Fig. 10. A total of 50.7 bonds were split in the 16-h period. The rate from 1 to 6 h was 4.0 bonds per h and 1.5 bonds per h from 8 to 16 h. The results show an initial relatively rapid reaction (*i.e.* 0 to 1 h) possibly due to the

splitting of the bonds responsible for complex formation, followed by a slower reaction which is probably representative of the digestion of the inhibitor (α_2-macroglobulin).

DISCUSSION

The anatomical, biochemical and physiological similarity of man and swine is generally accepted. With the increasing use of swine in biomedical research[24], any basic data which relates to man and swine should be of considerable interest and value to those workers engaged in the usage of swine as a laboratory animal. This laboratory in a previous communication[12], has described the general chromatographic and gel filtration properties of swine serum as compared to human. The present "discussion" on the interaction of swine elastase and the elastase inhibitors of swine serum, therefore, can be viewed as an extension of the above comparative studies[12].

Human serum was found to contain two inhibitors of swine elastase, α_1-antitrypsin and α_2-macroglobulin[6]. The results of the present study by contrast show that swine serum contains three inhibitors of swine elastase. Of the three, only two show any similarity to the inhibitors found in human serum and only the α_2-macroglobulins appear to be true analogs. The α_0/ϱ_1-globulin of swine serum and α_1-antitrypsin are similar with regard to molecular weight (mol. wt α_0/ϱ_1-globulin, 72 200; mol. wt α_1-antitrypsin, 72 000, ref. 12). Both proteins possess a high electrophoretic mobility; however, the mobility of the α_0/ϱ_1-globulin is, significantly, the faster of the two ($-5.3 \cdot 10^{-5}$ cm$^2 \cdot$ V$^{-1} \cdot$ s^{-1} *vs* $-6.1 \cdot 10^{-5}$ cm$^2 \cdot$ V$^{-1} \cdot$ s^{-1}). α_1-Antitrypsin is the principal elastase inhibitor of human serum whose concentration judging from the areas of the zones of undigested elastin produced in side-by-side experiments (as in Fig. 1) with human and swine serum, is about 3-fold greater than the α_0/ϱ_1-globulin of swine serum.

Judging from the zones of undigested elastin in Figs 1 and 2, the β_1-globulin is the principal elastase inhibitor of swine serum. Also, of special interest with regard to the β_1-globulin is the fact that this protein has no human serum counterpart. Further, it is of interest to note that swine trypsin is either very loosely bound by the β_1-globulin or is not inhibited at all (Fig. 1C). This finding suggests that the β_1-globulin is uniquely specific for elastase.

It was previously shown that 1 ml of human serum was capable of binding 0.9 mg of swine elastase and that the dissociation constant for the elastase–inhibitor complexes was $2.6 \cdot 10^{-9}$ (ref. 6). Swine serum also proved to be different from human serum in regard to these parameters. The results showed that 1.0 ml of swine serum was capable of binding 0.3 mg less elastase than human serum and also that the inhibitor–elastase complexes dissociated about 10-fold more readily than their human serum counterparts.

A property which shows no interspecies variation is the ability of elastase to slowly degrade the inhibitors following electrophoretic separation of elastase–inhibitor complexes and prolonged incubation at 37 °C (Fig. 3, ref. 6).

A method for the isolation of human α_2-macroglobulin from human serum has been described in a previous communication from this laboratory[14]. The present report shows that α_2-macroglobulin from swine serum can be isolated using, without variation, the same method. The α_2-macroglobulin from swine serum like the human

protein is partially denatured by dialysis against distilled water followed by lyophilization. Also, when lyophilized α_2-macroglobulin is subjected to analysis in the analytical ultracentrifuge, a 25-S component is seen. These findings parallel those obtained with human α_2-macroglobulin. Therefore, like human α_2-macroglobulin[14], lyophilization of this protein should be avoided.

Data has been presented in Figs 8–10 and Table II which confirm that swine α_2-macroglobulin, like its human analog[14], is both an inhibitor and a substrate of elastase. Also, like its human serum analog, swine α_2-macroglobulin is capable of binding approximately 1 mole of elastase. The dissociation constants for swine and human α_2-macroglobulin were respectively: $4.5 \cdot 10^{-9}$ and $1.3 \cdot 10^{-8}$ (ref. 14). Therefore, the human α_2-macroglobulin–elastase complex dissociated about 3-fold more readily than the swine α_2-macroglobulin–elastase complex, indicating that human α_2-macroglobulin has less affinity for the enzyme. It is likely that these results are due to subtle species differences such as conformation of the α_2-macroglobulin molecule.

The pH-stat and peptide-mapping data show with certainty that α_2-macroglobulin is digested by elastase. The pH-stat data show that the digestive process begins immediately upon the addition of elastase (Fig. 10). The relatively rapid production of protons in the initial phase of the reaction is likely due to complex formation showing that peptide-bond cleavage is an integral part of the reaction. These results lend support to the views of Ozawa and Laskowski[25].

Further work on the isolation and characterization of the fragments produced in the digestion of the α_2-macroglobulin–elastase complex and upon the isolation and characterization of the α_0/ϱ_1- and β_1-globulin inhibitors are in progress.

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