Degradation of Serum Amyloid A and Apolipoproteins by Serum Proteases†

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ABSTRACT: We have investigated the protease activity, present in human serum, that digests the serum amyloid A (SAA) protein. SAA radiolabeled with ^{125}I was incubated at 37 °C with serum and plasma and analyzed for degradation products by alkaline urea–polyacrylamide gel electrophoresis and gel filtration chromatography. Serum initially digested SAA to intermediates of 3000–5000 in molecular weight, and these were further degraded to smaller peptides with prolonged incubation. SAA was not degraded by plasma anticoagulated with ethylenediaminetetraacetic acid (EDTA) or heparin. Recalcification of plasma anticoagulated with EDTA led to the generation of protease activity against SAA whereas EDTA plasma defibrinated with thrombin was inactive. We employed both nonselective and selective protease inhibitors and synthetic substrates for kallikrein and plasmin to further characterize the serum protease. These studies demonstrated that degradation of SAA is not directly attributable to enzymes involved in coagulation, kinin formation, or fibrinolysis, but the unidentified protease may be activated by one of the clotting factors. The specificity of the SAA degradation was demonstrated in experiments with three of the well-characterized apolipoproteins. Apolipoproteins A-I, C-I, and C-III-1, which also associate with the plasma high-density lipoproteins, were not degraded by serum although they were good substrates for purified thrombin and plasmin.

Serum amyloid A (SAA)[1] is an acute phase reactant (McAdam et al., 1978; Rosenthal & Franklin, 1975; Gorevic et al., 1976) that is transported in serum in association with the high-density lipoproteins (HDL) (Benditt & Eriksen, 1977; Skogen et al., 1979; Benditt et al., 1979). SAA has a molecular weight of 11 500 (Franklin et al., 1975; Anders et al., 1975; Linke et al., 1975) and is thought to be the precursor of the M_r 8500 tissue protein of secondary amyloidosis, amyloid A (AA) (Linke et al., 1975). To date, there has been no direct evidence demonstrating a precursor–product relationship. However, several laboratories have reported that SAA is degraded to a protein of approximately the same size as AA by cells of monocytic origin (Lavie et al., 1978, 1980; Skogen et al., 1980a) and by enzymes present in serum (Skogen et al., 1980b; Skogen & Natvig, 1981).

We initially examined the ability of both serum and plasma to degrade SAA and found that only serum was active. We concluded from these data that the enzyme(s) involved in SAA degradation must be activated during coagulation. Coagulation is initiated by the activation of the Hageman factor (Ratnoff & Rosenblum, 1958; Ratnoff et al., 1961) as are fibrinolysis (Mandle & Kaplan, 1972; Collen, 1980) and kinin formation (Kaplan & Austin, 1971; Colman et al., 1981). Therefore, we conducted experiments to determine whether the SAA-degrading activity in serum was related to one or more of these pathways. Other apolipoproteins of HDL were also examined to determine if this enzyme activity is specific for SAA.

Materials and Methods

Materials

SAA-rich plasma was obtained from patients with chronic disease undergoing exchange plasmapheresis. We have previously described in detail the isolation of six polymorphic forms of SAA (Bausserman et al., 1980). SAA_4, one of the two quantitatively major forms of SAA, was used for most of these studies. SAA_1, which differs from SAA_4 in its electrophoretic mobility and solution properties, was also used in some experiments. Apolipoprotein A-I (apoA-I), apoC-I, and apoC-III-1 were isolated from plasma of normal subjects or patients with type 5 hyperlipoproteinemia by established techniques (Herbert et al., 1978). Fresh serum or plasma from normal volunteers was used as a source of SAA-degrading activity in the incubation experiments. Thrombin, plasmin, and clotting factor Xa were purchased from Sigma Chemical Co., and urokinase was from Abbott Laboratories. Diisopropyl fluorophosphate (DFP) and aprotinine were obtained from Calbiochem, antithrombin III was from Boehringer-Mannheim Biochemicals, heparin was from Riker Laboratories, and ϵ-aminocaproic acid (EACA), benzamidine, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and kaolin were from Sigma. The synthetic substrates for plasmin, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide (S2251), and for kallikrein, H-D-propyl-L-phenylalanyl-L-arginine-*p*-nitroanilide (S2302), were obtained from Helena Laboratories.

Methods

Radiolabeling. Proteins were radiolabeled with $Na^{125}I$ or $Na^{131}I$ (New England Nuclear) by using *N*-chlorobenzenesulfonamide sodium salt on derivatized nonporous polystyrene beads (Iodobeads, Pierce Chemical Co.). Protein (50 μg) dissolved in 0.1 mL of sodium phosphate buffer, pH 7.4, was added to the isotope. The reaction was initiated by the addition of one bead and terminated by its removal after 1 h at room temperature. Tracer was diluted with 2 mL of heat-inactivated (1 h at 60 °C), lipoprotein-free serum and dialyzed exhaustively to remove unreacted $^{125}I^-$. The radioactivity was 97–99% precipitable with trichloroacetic acid. The specific activity of the tracer ranged from 2.2 to 4.8 mCi/mg, and approximately 0.1 μCi was added to each incubation tube.

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[1] Abbreviations: SAA, serum amyloid A; AA, amyloid A; HDL, high-density lipoproteins; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; EACA, ϵ-aminocaproic acid; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; apoA-I, apolipoprotein A-I; apoC-I, apolipoprotein C-I; apoC-III-1, apolipoprotein C-III-1.

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Incubation Experiments. Tracer was added to 0.15–0.20 mL of serum or plasma and incubated at 37 °C for the indicated time periods. Aliquots of 0.05 mL were delipidated with 8 mL of methanol–diethyl ether (1:3). The protein precipitate was washed once with diethyl ether, dried under a stream of nitrogen, and redissolved in 0.5 mL of 0.1 M ammonium hydroxide. Twenty microliters was then analyzed by polyacrylamide gel electrophoresis.

Inhibitors were added to serum prior to the addition of tracer to give the following final concentrations: DFP, 12 mM; EACA, 0.75 M; benzamidine, 0.2 M; TPCK, 0.5 mM; TLCK 1.3 mM; aprotinine, 2000 units/mL (trypsin inhibitor units); antithrombin III, 100 units/mL (1 unit is the activity in 0.1 mL of normal serum); heparin, 100 units/mL; EDTA, 0.17 M. Buffer was added to controls to correct for volume changes. Dilution of serum did not affect the activity. Purified enzymes, plasmin (1 unit), thrombin (1 unit), and factor Xa (0.4 unit) were added immediately before tracer to 0.15 mL of serum, heat-inactivated serum (60 °C for 1 h), or saline.

Polyacrylamide Gel Electrophoresis. Alkaline urea–polyacrylamide gel electrophoresis was performed as described by Reisfeld & Small (1966). Acrylamide concentration in the separating gel was 7.5%, and the urea concentration was 8 M. Immediately after electrophoresis, gels were sliced as indicated in Figure 1, and the radioactivity in each slice was determined in a Micromedic Systems MS588 gamma counter. Results were expressed as percent of the total counts recovered from the gel. Radioactivity losses during organic solvent extraction, resolubilization and polyacrylamide gel electrophoresis were less than 10%.

Gel Chromatography. Delipidated serum samples were chromatographed on a 1.2 × 150 cm column of Sephadex G-100 eluted with 5 M guanidine hydrochloride. Fractions of 5 mL were collected, and the radioactivity in each fraction was determined. ApoA-1 (M_r 28 000), apoC-III-1 (M_r 10 000), reduced and carboxymethylated apoA-II (M_r 8500), and glucagon (M_r 3500) were used as molecular weight markers.

Activation of Hageman Factor. Kaolin, 0.5 mg/mL, was added to plasma and incubated 20 min at room temperature. Kaolin was removed by centrifugation at 2000 rpm for 20 min at 10 °C.

Assay of Kallikrein and Plasmin. Chromogenic substrates S2302 and S2251 were used respectively to assay kallikrein and plasmin activities in serum and plasma. Reaction mixtures containing 0.6 mM S2251 or 0.4 mM S2302 in 0.15 M NaCl–0.005 M Tris-HCl, pH 7.6, were preincubated at 37 °C in a thermostated cuvette. The reaction was initiated by addition of 0.2 mL of serum or plasma, giving a final volume of 2.0 mL. Changes in optical density at 405 nm were monitored in a Hitachi Model 100-60 spectrophotometer for 10 min. Activities were expressed as ΔA per minute.

Results

Incubation of SAA with Plasma. SAA_4 radiolabeled with ^{125}I was added to plasma and incubated at 37 °C for 20 h. Aliquots were delipidated and analyzed by alkaline polyacrylamide gel electrophoresis (Figure 1) as described under Methods. There was no change in the electrophoretic distribution of tracer when SAA_4 was incubated in plasma anticoagulated with either EDTA or heparin. Initiation of clotting by the addition of thrombin did not generate SAA_4 degrading activity. However, when EDTA plasma was recalcified and permitted to clot, the defibrinated plasma was capable of degrading SAA (Table I). Radioactivity was reduced from 59 to 20% in zone 3 and increased from 12 to 55% in the bottom third of the gel (zones 5 and 6). These observations indicated that platelets and other formed elements in the blood were not necessary for the generation of the SAA-degrading activity. They also suggested that coagulation may initiate this process.

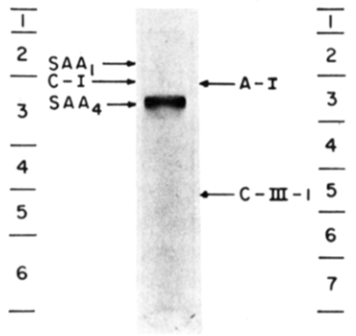

FIGURE 1: Alkaline urea–polyacrylamide gel electrophoresis of SAA_4. The positions where the gels were sliced for analysis of apoA-I and apoC-III-1 radioactivity are indicated on the right; bands for SAA_1, SAA_4, and apoC-I are on the left.

Table I: Time Course of SAA_4 Degradation by Serum and Plasma

	% radioactivity								
	in serum at time (h)						in plasma at time (h)		in plasma + Ca^{2+} at 24 h[a]
gel zone	0	1	3	6	15	24	0	24	
1	2	1	1	2	2	1	1	1	1
2	11	11	10	8	8	7	10	10	8
3	76	55	37	31	20	15	59	59	20
4	7	21	29	26	24	22	18	21	17
5	2	7	13	19	27	26	8	7	22
6	1	5	10	14	20	30	4	3	33

[a] Plasma anticoagulated with EDTA was recalcified and defibrinated before addition of SAA.

Time Course of SAA_4 Degradation by Serum. [^{125}I]SAA_4 was exposed to fresh serum for varying periods up to 24 h. Initially, 76% of the radioactivity was located in zone 3 and only 3% in zones 5 and 6 (Table I). With increasing incubation periods, the amount of radioactivity in zone 3 decreased, that in zone 4 remained relatively constant after an initial increase, and the radioactivity in zones 5 and 6 progressively increased. After 24 h, 15% of the radioactivity was recovered in zone 3 and 56% in zones 5 and 6. This digestion of SAA, moreover, does not appear due to the fact that the lipid-free protein rather than lipoprotein-bound SAA was used in these experiments. In separate studies, radiolabeled SAA was added to HDL, the lipoprotein-bound tracer was isolated by ultracentrifugation, and the tracer was incubated with serum. After 20 h of incubation, the fraction of radioactivity in zone 6 increased from 4 to 34%.

When serum was preincubated for 24 h before the addition of [^{125}I]SAA_4 and then further incubated for another 24 h, the radioactivity in zone 3 was reduced to 35%, and that in zones 5 and 6 increased to 30%. Therefore, while digestion of SAA_4 was most rapid during the early part of the incubation (Table I), degradation still occurred after 24 h, suggesting that the proteolytic activity either was very stable or was being continuously generated and deactivated throughout the incubation period.

Gel chromatography (Figure 2) was used to estimate the molecular weights of the degradation products. At time zero there was a small void volume peak followed by the major SAA peak, with little radioactivity in lower molecular weight

Table II: Effect of Inhibitors on SAA_4 Degradation by Serum[a]

	% radioactivity with the following inhibitor								
gel zone	none	EACA	aprotinine	benzamidine	TLCK	TPCK	DFP	EDTA	heparin and antithrombin III
1	1	1	1	1	1	1	1	1	1
2	10	8	9	11	9	10	11	8	9
3	19	52	28	62	39	27	58	26	20
4	19	26	26	18	23	27	18	32	23
5	27	8	19	5	12	19	6	18	22
6	24	6	18	4	17	17	5	15	25

[a] Tracer and sera incubated at 37 °C for 24 h. The concentrations of inhibitors are indicated in the text.

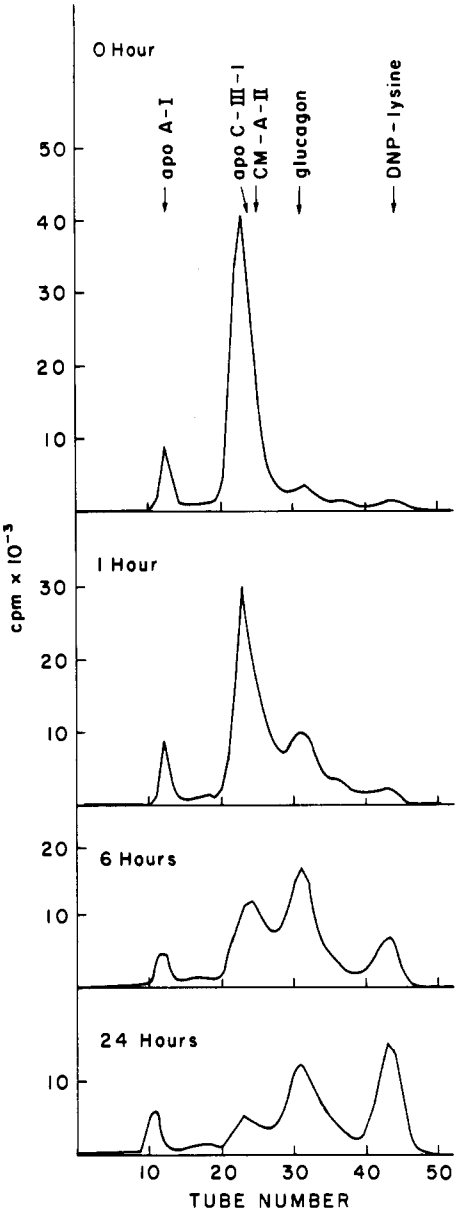

FIGURE 2: Gel chromatography of [^{125}I]SAA after incubation at 37 °C with serum for 0, 1, 6, and 24 h. A 1.2 × 150 column of Sephadex G-100 was eluted with 5 M guanidine hydrochloride. ApoA-I (M_r 28 000), apoC-III-1 (M_r 10 000), carboxymethylated apoA-II (M_r 8500), glucagon (M_r 3500), and DNP-lysine were used as molecular weight markers.

fractions. During incubation, peaks appeared first at a position corresponding to a molecular weight of 3000–5000 and later at the salt volume. The radioactivity eluting in these peaks increased throughout the incubation period while that in the SAA peak decreased. When the salt volume peak was analyzed by polyacrylamide gel electrophoresis, approximately

Table III: Degradation of SAA_4 by Serine Proteases[a]

	% radioactivity with the following addition			
gel zone	none	thrombin	plasmin	factor Xa
1	1	1	0	1
2	13	17	5	9
3	33	19	11	36
4	23	16	6	21
5	13	14	7	16
6	18	34	70	18

[a] SAA and enzymes incubated in serum at 37 °C for 20 h. Enzyme concentrations are indicated under Methods.

70% of the radioactivity was recovered in zone 6, suggesting that the radioactivity observed in this region of the gel reflected the accumulation of small peptides. The apparent molecular weight of the major intermediate was considerably smaller than amyloid A (M_r ~9000).

Effect of Protease Inhibitors. Eight compounds known to inhibit the enzymes of the coagulation and fibrinolytic systems were analyzed for their effects on the SAA-degrading activity in serum (Table II). SAA degradation was inhibited by DFP, indicating that it is a serine protease. Benzamidine, known to inhibit thrombin, plasmin, trypsin, and complement (Coats, 1973; Andrews et al., 1978), and EACA, an inhibitor of plasmin, also prevented SAA degradation. Heparin and antithrombin III, which inhibit kallikrein (Lahiri et al., 1976) and several of the clotting factors (Stead et al., 1976; Rosenberg & Damus, 1973), did not inhibit SAA degradation, nor did aprotinine, another inhibitor of kallikrein (Colman & Bagdasarian, 1976). TPCK and TLCK, inhibitors of chymotrypsin and trypsin, respectively, similarly failed to block SAA proteolysis.

In a separate experiment the effect of adding EACA to blood before coagulation was tested. Whole blood was drawn into a plastic syringe and immediately transferred to tubes containing solutions of EACA to yield final concentrations of 0.15, 0.30, and 0.75 M. Clot formation was not inhibited by EACA although it was slower at the highest concentration. SAA degradation was completely inhibited at all three concentrations of EACA, suggesting that the enzymes of the coagulation cascade do not directly act on SAA_4.

Cleavage of SAA_4 by Serine Proteases. Three enzymes that are activated during coagulation were tested for their ability to degrade SAA_4. Thrombin and plasmin increased the digestion of SAA_4 when added to serum (Table III) and demonstrated a similar degree of activity when incubated with SAA_4 in heat-inactivated serum (60 °C for 1 h) or saline (data not shown). Addition of clotting factor Xa to serum did not enhance the effect seen with serum alone (Table III), and no digestion was observed when factor Xa was incubated with SAA_4 in heat-inactivated serum or saline.

Synthetic substrates were used to compare the activities of kallikrein and plasmin in fresh serum and plasma. Kallikrein

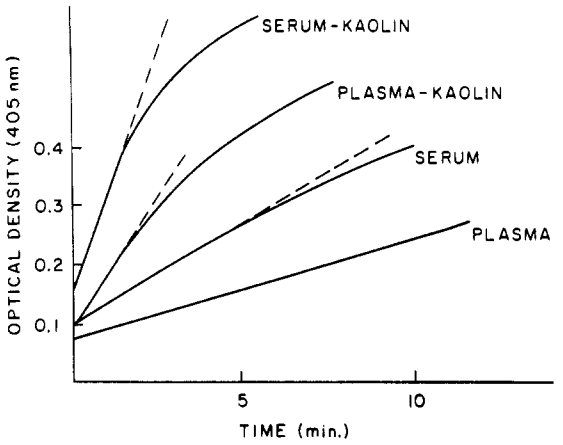

FIGURE 3: Assay of kallikrein with chromogenic substrate S2303. Substrate was incubated with 0.2 mL of serum, plasma, serum treated with kaolin (0.5 mg/mL), or plasma treated with kaolin (0.5 mg/mL) at 37 °C and the absorbance monitored at 405 nm.

Table IV: Degradation of SAA_4 by Urokinase-Treated Plasma[a]

	% radioactivity at time (h)						
gel zone	0	1	3	6	12	20	20[b]
1	1	0	0	0	0	0	0
2	10	7	6	7	8	7	12
3	59	27	25	24	25	21	55
4	18	17	19	18	23	21	22
5	8	17	14	14	16	17	7
6	4	33	35	37	28	31	4

[a] Urokinase (2500 units/mL) added to plasma, which was then incubated at 37 °C. [b] Aprotinine (2000 units/mL) added with urokinase and incubation continued for 20 h.

activities, expressed as ΔA per minute were 0.028 and 0.014 min^{-1} in serum and plasma, respectively (Figure 3). Since kallikrein is activated by the Hageman factor, kaolin was added to plasma to maximally activate the Hageman factor, and this increased the activity of kallikrein to 0.074 min^{-1}, a value well above that observed in serum. However, the kaolin-treated plasma did not digest SAA_4 during a 24-h incubation. This observation suggests that the SAA_4-degrading activity in serum is not due to the activation of kallikrein during coagulation.

The activity of plasmin in both serum and plasma was 0.003 min^{-1} and remained at the same low level during 5 h of incubation at 37 °C. When urokinase, an activator of plasmin, was added to EDTA plasma, activity against the synthetic substrate increased 10-fold to 0.029 min^{-1}, and SAA-degrading activity was generated (Table IV). Urokinase in saline or heat-inactivated serum was inactive against SAA, indicating that SAA_4 digestion was mediated by an enzyme other than urokinase. The time course of the reaction in urokinase-treated plasma, however, was quite different from that observed with serum. When SAA_4 was incubated in serum, reaction products accumulated gradually over a period of 24 h. In contrast, maximum digestion was obtained during the first hour of incubation with urokinase-treated plasma, after which there was no further increase in low molecular weight products. Moreover, the enzyme activity in plasma activated by urokinase was totally inhibited by the addition of aprotinine, which had no effect on the serum enzyme. It was possible that an α_2-macroglobulin–plasmin complex that resisted aprotinine inhibition was responsible for the SAA proteolysis. To test this possibility, plasma was incubated with streptokinase for 0, 15, 30, or 60 min before the addition of aprotinine. Tracer was added after 60 min and analyzed for degradation products

Table V: Degradation of Apolipoproteins by Serum and Serine Proteases[a]

		% radioactivity with the following treatment					
		serum		thrombin		plasmin	
	gel zone	0 h	20 h	serum	buffer	serum	buffer
apoA-I	1	1	1	0	4	1	0
	2	13	16	20	16	5	5
	3	66	60	28	20	12	7
	4	13	13	18	12	9	16
	5	5	5	15	8	17	22
	6	2	3	8	13	7	20
	7	1	3	11	27	49	29
apoC-III-2	1	0	1	0	2	1	0
	2	5	7	7	8	6	3
	3	7	8	8	6	9	4
	4	14	11	11	7	7	6
	5	56	55	58	31	10	19
	6	13	12	11	20	23	23
	7	5	6	5	27	43	45
apoC-I	1	1	0	ND	ND	0	0
	2	10	4			6	4
	3	54	52			33	12
	4	19	27			16	4
	5	6	6			6	2
	6	5	6			8	8
	7	5	5			32	70

[a] All incubations were at 37 °C for 20 h except for the 0-h serum samples.

after an additional 20-h incubation. Aprotinine completely inhibited degradation by streptokinase-treated plasma regardless of when it was added to the incubation mixture. In addition, serum passed over a lysine–agarose affinity column, which removed all plasminogen, fully retained its ability to degrade SAA. These results indicate that plasmin is neither the protease in serum that degrades SAA nor the activator of the protease.

Specificity of SAA Degradation. Another polymorphic form of SAA, SAA_1, which differs from SAA_4 in its solution properties and electrophoretic mobility (Bausserman et al., 1980), was also degraded by serum and the reaction followed a similar time course (data not shown). Three other HDL apoproteins, A-I, C-1 and C-III-1, were also radiolabeled with ^{131}I or ^{125}I and incubated with serum. The electrophoretic distributions of these proteins were not altered by incubation in serum for 24 h (Table V), and the column chromatographic elution profiles were identical before and after incubation (not shown).

The effects on these apoproteins of purified enzymes in the presence and absence of serum were also examined (Table V). Thrombin degraded apoC-III-1 in saline but not in serum. ApoA-I was digested in serum containing added thrombin, but a greater effect was seen when serum was omitted from the incubation mixture. Addition of plasmin to either serum or saline resulted in marked degradation of both apoC-III-1 and apoA-I. Plasmin appeared to be inactive against apoC-I in serum but degraded apoC-I in saline. It appears that these proteins are substrates when purified preparations of both enzyme and protein are used. However, the apolipoproteins are not degraded by serum, possibly because of competition by other substrates or lower concentrations of active enzyme.

Discussion

Several laboratories have reported the presence in serum of a factor capable of degrading amyloid A fibrils (Kedar et al., 1974, 1982; Skogen & Natvig, 1981; Wegelius et al., 1982)

and SAA (Skogen et al., 1980a; Skogen & Natvig, 1981). The potential importance of this amyloid-degrading factor was underscored by reports of reduced activity in patients with chronic inflammatory diseases with (Wegelius et al., 1982; Maury & Teppo, 1982; Kedar et al., 1982) and without (Maury et al., 1982) amyloidosis. It has not been generally appreciated that plasma, unlike serum, does not contain SAA-degrading activity.

Like Skogen & Natvig (1981), we observed that several serine proteases found in serum degrade SAA; however, using synthetic substrates and a variety of inhibitors, we were able to exclude kallikrein, plasmin, and the enzymes of the coagulation cascade as the protease active in serum. Skogen & Natvig (1981) described formation of an intermediate degradation product with a molecular weight similar to that of AA after incubation of SAA with pancreatic kallikrein, elastase, collagenase, plasmin, and thrombin. We did not analyze the products of digestion after very short incubation times. However, there was no evidence of a 9000-dalton intermediate in the column chromatographic profile after a 1-h incubation with serum (Figure 2). Rather, most of the SAA was initially cleaved to peptides of M_r 3000–5000, and these were subsequently digested to smaller peptides.

The relative specificity of the serum SAA-degrading activity is the principle evidence suggesting a possible role in SAA catabolism. Other apolipoproteins transported in HDL were not degraded during incubation with serum, although in simple solution all were substrates for many of the serine proteases that digested SAA. It should be noted that SAA and other apolipoproteins readily reassemble with HDL when added to serum (L. L. Bausserman and P. N. Herbert, unpublished results), but association with lipid apparently does not protect SAA from proteolytic digestion.

These studies illustrate the difficulties that will be encountered in attempts to isolate the SAA protease in serum. A number of serine proteases present in serum degrade SAA in buffered salt solutions but do not do so in serum, possibly because of the presence of natural inhibitors or competition by other substrates. It should also be noted that although thrombin, plasmin, and kallikrein do not account for SAA digestion in serum, this does not exclude their potential importance in SAA or AA proteolysis in vivo. The activity of plasmin in urokinase-activated plasma is particularly noteworthy since the SAA-degrading capacity of the fibrinolytic system is very active in vitro.

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Registry No. Proteinase, 9001-92-7.

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