The Proteolytic Enzyme System of Skin. VII. Effect of Immune Reactions.* (28248)

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The participation of proteolytic enzymes in the allergic phenomenon has been postulated for many years. Indeed, it has been demonstrated that proteolytic enzymes can produce reactions apparently identical to those brought about by the interactions of antigens with their specific antibodies(1). This has led to the hypothesis that proteolytic enzymes are activated as a consequence of an antigen-antibody reaction. Experimental evidence bearing on the validity of this concept has been contradictory(2,3). In general, most of the experiments concerned with the effect(s) of antigen-antibody reactions on the activation of proteolytic enzymes have been conducted with crude and often poorly characterized enzyme systems. The use of a better defined enzyme system which is present in extracts of rat skin(4-9), would favor a more critical evaluation of the effect(s) of antigen-antibody reactions on proteolytic enzymes.

In these extracts of rat skin, 4 distinct enzymes, designated Proteinase A, the A_1-esterase, the A_2-esterase, and Proteinase C, have been characterized, partially purified, and in some cases separated from each other. With the exception of Proteinase C, which hydrolyzes casein, synthetic substrates are known for these enzymes. Most of these enzymes appear to exist in an inactive state, thus the possibility exists that they can be activated by antigen-antibody reactions.

The present paper is concerned with the interrelationship of antigen-antibody reactions and the proteolytic enzyme system of rat skin. Both *in vivo* and *in vitro* studies have been performed.

Materials and methods. Preparation of skin extracts. Fresh rat abdominal skin was minced and extracted at 1°C with 4 ml of KCl solution/g of skin mince(9). Various types of extractions are shown in Table I. Supernatant solutions obtained after centrifugation at 25,000 × *g* for 30 minutes at 1°C served as enzyme sources. *Amino acid esterase assays.* Proteinase A, in addition to its ability to hydrolyze casein, will also hydrolyze acylated and non-acylated aromatic amino acid esters such as N-acetyl-L-tyrosine ethyl ester (ATEE)‡ and TEE. The specificity of the A_1- and the A_2-esterase is not as inclusive, each possessing one facet of the substrate specificity of Proteinase A. Thus, the A_1-esterase will hydrolyze only *acylated* aromatic amino acid esters, *e.g.*, ATEE, and conversely only *non-acylated aromatic* amino acid esters, *e. g.*, TEE, are hydrolyzed by the A_2-esterase. Despite the overlapping specificity patterns of Proteinase A, the A_1-esterase, and the A_2-esterase, the activity of each in a mixture can be determined by a differential assay procedure using ATEE, TEE and a specific inhibitor, AIn, isolated from sheep serum(7,8). Amino acid esterase activity was determined at 30°C in reaction solutions at ionic strength 0.6 using ATEE and TEE as substrates(5,7). Hydrolysis of ATEE and TEE was measured at pH 8.0 and 6.5, respectively. Incubation of enzyme extracts with the AIn preparation to inhibit Proteinase A was conducted under standardized conditions(5). Esterase activity was expressed as the zero order reaction constant, k_o, defined as μmoles of substrate hydrolyzed/minute/ml of extract. *Proteinase assays.* Most of the proteolytic activity of rat skin extracts can be ascribed to Proteinase C(4,6). In such extracts, Proteinase C can undergo re-

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‡ TEE, L-tyrosine ethyl ester; Ea, egg albumin; PCA, passive cutaneous anaphylaxis; Ag, antigen; Ab, antibody.

TABLE I. Amino Acid Esterase and Proteinase Activities of Skin Extracts After PCA.*

Group†	Treatment	mg protein per ml extract	(k_o) ATEE Total	(k_o) ATEE With AIn	(k_o) TEE	Proteinase activity × 10^3
			(I)	(II)	(III)	(IV)
I	Control	8.63	1.66	.88	.20	62
	PCA	8.79	1.64	.88	.19	64
II	Control	7.13	1.21	.97	.19	14
	PCA	7.22	1.18	.98	.20	16
III	Control	7.17	.60	.60	.20	8
	PCA	7.06	.58	.64	.21	7
IV	Control	8.81	1.64	.82	.18	63
	PCA	9.06	1.62	.88	.19	64

* Data of column II are a measure of the A_1-esterase. Data of column III, corrected for Proteinase A activity, are a measure of A_2-esterase. The difference between columns I and II represents the activity of Proteinase A.

† Groups I, II and III utilize skin extracts obtained after extracting skin for 15 min with 1.6 M KCl, 0.8 M KCl, and 0.4 M KCl, respectively. In these groups, animals were sacrificed 60 min after injection of antigen. Animals of group IV were sacrificed 15 min after injection of antigen and skin extracts obtained by extraction for 15 min with 1.6 M KCl.

versible inactivation; a time-dependent process proceeding optimally in solutions of ionic strength 0.6-0.8(4). The reverse or activation reaction is essentially instantaneous upon exposure of the inactivated extract to ionic strength 1.4 or greater(4). This behavior was shown to be due to the ionic strength-dependent association and dissociation of Proteinase C with a skin inhibitor, CIn. Both components have been separated from each other and studied in some detail(6). In the *in vivo* experiments described here, aliquots of rat skin extracts obtained immediately after centrifugation were mixed with phosphate buffer-KCl solutions(4) so that the final ionic strength was 0.8 and phosphate buffer concentration 0.05 M at pH 7.5. Alkali-denatured casein was mixed with diluted skin extract so that the enzyme-substrate reaction solution was at pH 7.5, ionic strength 0.8, and phosphate buffer concentration 0.05 M. The time interval between centrifugation of extracts and addition of casein was approximately 10 minutes. Casein digestion was carried out for 30 minutes at 35°C before addition of trichloroacetic acid. Optical densities of the supernatant solutions were determined at 280 mμ. Proteinase activity was expressed as the optical density change at 280 mμ/minute/ml of original skin extract as determined under standard conditions(4). Protein was determined by the method of Lowry *et al.*(10) using a commercial protein standard.§ *Preparation of proteinase C and CIn.* Proteinase C for *in vitro* studies was prepared as previously described(6) except that fresh rat skin rather than acetone-dried skin served as the starting material. The skin extract was dialyzed against water and the resulting precipitate taken up in 0.6 M KCl to give the EDP-1 fraction. Fraction EDP-1 was treated with alumina gel and after removal of the supernatant solution, the precipitate was eluted with 0.1 M phosphate buffer at pH 7.5 containing 2.93 M KCl to give the SAL-3.2 fraction. Fraction SAL-3.2 contained 159 × 10^{-3} proteinase units and 0.80 mg of protein/ml. This represented a 23-fold purification in a yield of 42%. The proteinase activity of Fraction SAL-3.2 was the same whether it was exposed to ionic strengths 1.6 or 0.8 for several hours before assay. Thus, this Proteinase C preparation was free of its inhibitor. CIn was prepared from fresh rat skin by a previously described method(6). The CIn preparation contained only a trace of proteinase activity. One ml of this preparation inhibited 1 ml of the SAL-3.2 fraction maximally upon incubation for 4 hours at ionic strength 0.8 and 1°C. The maximum inhibition of proteinase activity of Fraction SAL-3.2 by CIn under these conditions never exceeded 45%. Studies with a more purified

§ Protein Standard, Ampagent, Aloe Scientific.

Proteinase C preparation revealed that maximum inhibition of proteinase activity by CIn approached 60 to 65%(6). The reason(s) for this maximal but incomplete inhibition remains obscure(6). Therefore, in this paper, maximum inhibition of the activity of Fraction SAL-3.2 by CIn is understood to be 45%. *Preparation of rabbit anti-egg albumin sera.* Young adult albino rabbits were immunized against Ea‖ treated with alum in the manner described by Kabat and Mayer(11). One week after the final injection of antigen, as much blood as possible was collected by cardiac puncture. The blood was allowed to clot and the resulting sera were tested qualitatively for anti-Ea protein(12). Those sera exhibiting a high anti-Ea level were pooled, passed through a Seitz filter, and merthiolate added as a preservative(13). The immune serum contained 467 μg of anti-Ea N/ml as determined by quantitative antibody N determinations(14). The serum was stored in small volumes in the frozen state. *Preparation of antigen-antibody aggregates.* Ag-Ab‡ aggregates were prepared by adding 1 ml of rabbit anti-Ea serum, containing 467 μg of Ab N/ml, to 1 ml of an Ea solution in 0.9% NaCl containing 67.8 μg of N/ml. This ratio of Ab N to Ag N was found by experimental trial to be the combining ratio of the reactants at the equivalence point. After incubation at 35°C for 30 minutes, the solutions were stored overnight in the refrigerator. The precipitates, obtained by centrifugation at 850 $\times$ *g* in the refrigerated centrifuge for 30 minutes, were washed twice with 2 ml portions of 0.9% NaCl. *Production of PCA.*‡ PCA was produced in albino rats by injecting anti-Ea sera intradermally and several hours later injecting intravenously Ea and Evans Blue dye(15). Those areas in the skin undergoing this type of immune response appeared colored within a few minutes indicating leakage of plasma into the tissue spaces.

Experimental and results. In vivo *studies.* PCA was produced on one side of the abdominal skins of 120 to 250 g male albino rats by placing 12 to 15 0.1 ml intradermal injections of anti-Ea serum diluted with 0.9% NaCl to contain 140 μg of Ab N/ml. The other side of the abdominal skin received the same number of injections of normal rabbit serum diluted in the same manner as the antiserum. Two hours later, each animal was injected intravenously *via* a tail vein with 1 ml of a solution consisting of equal volumes of Ea (3 mg/ml) and 1% Evans Blue dye, both made up in 0.9% NaCl. An intense blue coloration appeared on the side of the abdominal skin which received antiserum; the other side, injected with normal rabbit serum, was devoid of color. The animals were sacrificed 15 or 60 minutes later by decapitation and their abdominal skins removed, divided into PCA and control halves, and pooled. Extraction procedures and results of enzyme assays are shown in Table I. Data in this Table are averages of duplicate determinations, which agreed within 5%, of pooled skin from each group derived from 12 animals.

The results presented in Table I demonstrated no differences between any of the measured activities of control and PCA skin extracts within one given extraction procedure. It is also apparent that enzymatic activities of skin removed 15 or 60 minutes after injection of antigen were comparable (compare Groups I and IV, Table I). The extractant used for Group I, 1.6 M KCl, while optimal for extraction of the proteolytic enzyme system of rat skin, also permitted instantaneous activation of Proteinase C. Thus if Proteinase C were activated as a result of PCA, the experimental procedure used could not possibly detect such activation. This fact was realized but the protocol used was selected because the enzymatic activities of the other proteolytic enzymes in rat skin were optimally extracted with 1.6 M KCl. In an attempt to overcome this limitation placed by the above extraction procedure on the assay of Proteinase C, skin was extracted at ionic strengths 0.8 and 0.4. The possibility existed that any Proteinase C activated *in situ* as a consequence of PCA might be more extractable at these lower ionic strengths. Under these conditions Proteinase C would not be

‖ Egg albumin, recrystallized 5x, Pentex, Inc., Kankakee, Ill.

TABLE II. Proteinase Activity After Incubation of Ag-Ab Aggregates with Proteinase C Maximally Inhibited by CIn.*

Temp of incubation	Ag-Ab aggregate	Proteinase activity $\times 10^3$
1°	+	75
1°	—	73
35°	+	78
35°	—	72

* These data represent typical results obtained from experiments repeated at least 4 times.

activated during the extraction procedure. However, the data presented in Table I *cf.* Column IV for Groups II and III) indicated that no activation of proteinase activity occurred as a result of PCA.

In vitro *studies. Incubation of Ag-Ab aggregates with Proteinase C maximally inhibited by CIn.* Proteinase C (0.42 ml of Fraction SAL-3.2) was added to 0.42 ml of the CIn preparation, 0.08 ml of 0.1 M KCl, 0.58 ml of 4 M KCl, and water to a final volume of 4.80 ml at ionic strength 0.8. The resulting solution was incubated at 1°C for 4 hours. Maximal inhibition of Proteinase C occurred under these conditions. Two ml of this enzyme solution were added to an Ag-Ab aggregate and the mixture stirred thoroughly with a glass rod. The mixtures were incubated at 1°C or 35°C for 30 minutes and then centrifuged for 30 minutes in the refrigerated centrifuge at 850 $\times$ *g*. Immediately after centrifugation, 0.8 ml of the supernatant was added to a solution consisting of 1.2 ml of 0.8 M KCl, 5.0 ml of 0.07 M phosphate buffer, pH 7.5, containing 0.61 M KCl so that the final ionic strength was 0.8. Control experiments were performed in identical manner in the absence of Ag-Ab aggregates. Typical results of such experiments shown in Table II revealed no change in Proteinase C activity at either temperature of incubation. In some experiments, the inhibited enzyme was incubated at ionic strengths 0.31, 0.40 and 0.60 with Ag-Ab aggregates as described above. The results obtained with such studies were essentially identical with those shown in Table II. *Effect of* in situ *formation of Ag-Ab aggregates on Proteinase C maximally inhibited with CIn.* In these experiments, rabbit anti-Ea sera were mixed with Ea in the presence of Proteinase C maximally inhibited by CIn. Aliquots (0.07 ml) of Fraction SAL-3.2 were inactivated with CIn as described above. To these maximally inactivated Proteinase C solutions were then added 0.25 ml of rabbit anti-Ea sera (containing 117 μg of Ab N), 0.25 ml of Ea in 0.9% NaCl (containing 16.9 μg of N), 0.05 ml of 0.1 M KCl, 0.36 ml of 2.0 M KCl, and water to a volume of 2.0 ml. The final ionic strength was 0.8. These solutions were incubated at 35°C for 30 minutes before addition of 5 ml of 0.07 M phosphate buffer, pH 7.5, containing 0.61 M KCl so that the final ionic strength was 0.8. Two ml aliquots of the final solution were assayed with casein in the usual manner. Typical results of the effect of interaction of antigen and/or antisera with Proteinase C maximally inhibited by CIn are shown in Table III. In some experiments, interaction of antigen and antiserum was conducted at ionic strength 0.6. These results were identical with those shown in Table III. Data of Table III again indicated that no activation of Proteinase C, which had been maximally inhibited by CIn, occurred. Actually, proteinase activity was completely abolished after interaction of antigen with antiserum. In control experiments, however, where the separate effects of antigen and antiserum were tested, the same inactivating effect was observed upon the addition of antiserum, but not antigen, to the partially inactivated enzyme solution (*cf.* Table III). When antiserum and/or antigen was added to Fraction SAL-3.2 or to the CIn preparation prior to their combination, the same inactivating effect was observed with antiserum alone. However, normal rabbit serum also inactivated the caseinolytic activity of Proteinase

TABLE III. Proteinase Activity of Proteinase C Maximally Inhibited with CIn After *in situ* Formation of Ag-Ab Aggregates.*

Antigen	Antisera	Proteinase activity $\times 10^3$
+	+	0
—	—	70
+	—	72
—	+	0

* These data represent typical results obtained from experiments repeated at least 4 times.

C which had been maximally inhibited by CIn. Heating this serum at 55°C for 30 minutes destroyed its inhibitory activity. Thus, it is apparent that the inactivation by the immune serum is due to heat labile component(s) present in normal rabbit serum.

Discussion. The experiments described here indicate that antigen-antibody reactions do not activate any of the enzymatic activities of the proteolytic enzyme system of rat skin. In *in vivo* experiments where PCA was induced in the rat, control and PCA skin extracts exhibited the same enzyme activities when assayed against casein, ATEE, and TEE. Similarly, the results of *in vitro* experiments demonstrated that Ag-Ab aggregates had no effect upon Proteinase C in combination with its inhibitor, CIn. Thus, these results do not lend support to the hypothesis that proteolytic enzymes are activated as a result of antigen-antibody reactions. This conclusion, however, is limited to the proteolytic enzyme system of rat skin as described and a single antigen-antibody system, *viz.*, the egg albumin-rabbit anti-egg albumin system. One would have to investigate other species and other immune systems before a more definitive clarification of the effect(s) of antigen-antibody reactions on the activation of proteolytic enzymes could be stated.

On the other hand, Inderbitzin and Goetschmann(16) found a decreased proteolytic activity in PCA-rat skins when compared to that found in normal rat skin. These authors postulated that the decreased activity could be due to the *in situ* release of enzyme after antigen-antibody interaction or enzyme inhibition by an inhibitor released at the site of the PCA reaction. However, the proteolytic activity studied was different than that described here and the two systems cannot be compared.

Inderbitzin(17) later reported that exposure to passive cutaneous anaphylaxis produced no alteration in the proteolytic activity of an enzyme fraction extracted from rat skin.

Summary. Passive cutaneous anaphylaxis was induced in the rat using the egg albumin-rabbit anti-egg albumin system. Enzymatic activities of extracts of skin exposed to this immune reaction were assayed with casein, N-acetyl-L-tyrosine ethyl ester, and L-tyrosine ethyl ester as substrates. No difference existed between these extracts and those of control skin extracts. Purified Proteinase C was maximally inactivated by mixing the enzyme with its specific inhibitor. The inactivated enzyme solution was added to preformed antigen-antibody aggregates and no activation effect was observed. Similarly, no activation effect was found when the immune reaction was allowed to proceed *in vitro* in the presence of inactivated Proteinase C.

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