The gift of a sample of 6-dimethylamino-9-β-D-ribofuranosylpurine (IV) from Dr. HENRY KISSMAN of Lederle Laboratories is also gratefully acknowledged.

SUMMARY

Thymidine exists in the diketo form in deuterium oxide solution, and cytidine exists in the amino form. In acid solution, cytidine and cytidylic acid probably have an imonium structure. The monosodium salt of adenylic acid has the amino form in solution and, possibly, the imino form in the solid state. Adenylic acid has an imonium structure in the solid state.

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THE PROTEOLYTIC ENZYME SYSTEM OF SKIN

III. PURIFICATION OF PROTEINASE C AND ITS SEPARATION FROM AN INHIBITOR*

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The major portion of the proteolytic activity in high ionic strength extracts of rat skin acetone powder can undergo reversible inactivation upon exposure to an ionic strength of 0.6 to 0.8 for several hours[1]. The enzyme susceptible to such treatment was designated Proteinase C and its conversion from the inactive to the active state was readily attained by brief exposure to an ionic strength of 1.4 or greater. It was postulated[1] that the inactivation of Proteinase C occurred by association of the enzyme with an inhibitor, CIn, and that the reversal of inactivation was the result of Proteinase C–CIn

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dissociation. An effect of ionic strength was also noted on the rate of casein digestion by extracts containing Proteinase C in the activated state[1]. Casein was hydrolyzed optimally at approximately pH 7.5 and ionic strength 0.6 to 0.8. Above or below this ionic strength, the hydrolysis rate was diminished.

Acylated or non-acylated amino acid esters or dipeptide esters in which an aromatic amino acid was involved in the ester bond were also hydrolyzed by rat skin extracts[2]. The esterase activity was not due to Proteinase C and the responsible enzymes, A_1 and Proteinase A, were shown to have closely related substrate specificities. One notable exception was that the A_1 enzyme was unable to hydrolyze non-acylated aromatic amino acid esters such as L-tyrosine ethyl ester (TEE). The test substrate used for the determination of A_1 and Proteinase A was N-acetyl-L-tyrosine ethyl ester (ATEE) and their individual contribution to the hydrolysis of this substrate was measured with the use of an inhibitor, AIn, prepared from sheep blood[2]. AIn stoichiometrically inhibited only the activity of Proteinase A.

This paper will describe the purification of Proteinase C and its separation from the A_1 enzyme and Proteinase A. It will also be shown that Proteinase C, in the purified state, is unresponsive to changes in ionic strength prior to assay and that it will react with an inhibitor preparation, also described in this paper, whose properties fulfill the requirements for the postulated CIn inhibitor. A portion of this work has been described in a short communication[3].

MATERIALS AND METHODS

The preparation of rat skin acetone powder[1], ATEE[2], and the blood inhibitor, AIn[2], have been given.

Alumina gel was prepared according to HAWK, OSER AND SUMMERSON[4]. From the initial reaction mixture of 52 liters there was obtained 4 liters of a very fine gel suspension containing 18 mg solids per ml.

Proteinase[1] and esterase[2] activity was measured with casein and ATEE as substrates and protein nitrogen[2] was estimated as already described.

The terms *inactivation* and *activation conditions*[2] of assay, *i.e.* the exposure of an enzyme preparation to ionic strength 0.6 to 0.8 for approximately 6 hours at 1° or to ionic strength 1.6, respectively, refer only to treatment of the enzyme prior to addition to the casein substrate. The former procedure leads to inactivation of Proteinase C whereas the latter procedure instantaneously and completely reverses the inactivation[1,2]. Regardless of the ionic strength pre-treatment, the assay conditions in the presence of casein were constant.

All concentrations of various materials are reported per ml of enzyme-substrate assay solution.

EXPERIMENTAL AND RESULTS

As previously indicated, Proteinase C in initial skin extracts has been defined as an enzyme which can undergo an ionic strength-dependent, reversible inactivation. It was postulated that this inactivation occurred through the interaction of the enzyme with an inhibitor. Proof of this postulation necessitated preparation of an enzyme (Proteinase C), whose activity was unaffected by ionic strength prior to assay, and an inhibitor fraction whose interaction with the purified proteinase was reversible and ionic strength-dependent. The following preparative procedures were developed to achieve these objectives.

Fractionation procedure for Proteinase C

All procedures were carried out at 0° to 3° except that involving methanol precipitation where a temperature of —6° was maintained. The International refrigerated

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centrifuge was used for all separations and relative centrifugal force values are given as tip measurements. All fractions were assayed for both proteinase and esterase activity.

Step I. *Extraction.* Skin acetone powder (200 g) was extracted with 3000 ml 1.6 *M* KCl for 16 hours and the suspension centrifuged for 45 minutes at 3500 × *g*. The supernatant fluid, containing some floating fine skin particles, was filtered through a bed of glass wool supported on a porcelain filter disk to give Fraction E-1.6; volume 2400 ml. The skin residue was discarded.

Step II. *Dialysis.* Expanded $1^5/_8$ inch casings were pressure loaded with approximately 500 ml E-1.6 and *each* casing dialyzed with spinning, by attachment to a 45 r.p.m. synchronous motor, against 7 liters ion-exchanged water for 8 hours and continued for 16 hours against a fresh 7 liter volume. The precipitate, obtained by centrifugation of the dialyzed suspension at 3500 × *g* for 60 minutes, was washed with ion-exchanged water (0.08 ml per ml of E-1.6 dialyzed). The washed precipitate was then suspended with the aid of a very close-fitted leucite-glass homogenizer in 0.60 *M* KCl to give Fraction EDP-1 with a volume equal to 0.06 ml per ml E-1.6 dialyzed. This fraction is fairly viscous. The aqueous phase from the dialysis step, to which was added the water-wash of the precipitate, was stored at —25° *.

Step III. *Alumina gel adsorption.* One volume of alumina gel was mixed with 1.0 volume of 1.8 *M* KCl in a precipitating jar and 1.0 volume EDP-1 added. This gave an ionic strength of 0.8 and at the end of 30 minutes, including the time for the transfer to 100 ml centrifuge cups, the suspension was centrifuged for 30 minutes at 3500 × *g*. The supernatant fluid was decanted, saved, and the packed precipitates transferred equally to three 25 ml stainless steel centrifuge cups with 0.80 *M* KCl (0.40 ml per ml EDP-1 used) and thoroughly mixed. After centrifugation at 25,000 × *g* for 30 minutes, the washings were removed from the firmly packed precipitates and added to the supernatant fluid from the adsorption step to give Fraction SAL-0.8. This fraction contains A_1 plus Proteinase A activity.

Step IV. *Elution.* The precipitates from Step III were eluted with 0.1 *M* phosphate buffer containing 2.93 *M* KCl, pH 7.5 (ionic strength, 3.2), at a ratio of 0.35 ml per ml EDP-1 used and after thorough dispersion, centrifuged 45 minutes at 27,000 × *g*. The soluble material was carefully removed with a small bulb pipet and stored in a 100 ml graduate at —25°. The precipitates were re-extracted with the same solvent (0.15 ml per ml EDP-1 used) as before and the extract added to the same graduate to give Fraction SAL-3.2. The activity in this fraction is labile and should be used immediately for the next step or stored at —25°.

Step V. *Methanol precipitation.* The precipitation of activity in Fraction SAL-3.2 was performed at ionic strength 0.35, pH 7.5, and at a methanol concentration of 25 volume per cent. One volume of SAL-3.2 was rapidly added to a solution containing 8.1 volumes of ion-exchanged water and 3.0 volumes of methanol measured at —25°. The temperature of —6° was maintained for 20 minutes before centrifugation at 3500 × *g* for 70 minutes. The water-clear supernatant fluid was discarded and the precipitates suspended in 0.60 *M* KCl to give a final volume equivalent to 0.30 ml per ml SAL-3.2 used. The nearly clear solution was then transferred to a 25 ml stainless

* This fraction contains at least two types of esterase activity different from Proteinase A. One is similar or identical to the A_1 enzyme[2] and the other, A_2[5], hydrolyzes non-acylated aromatic amino acid esters such as TEE and L-phenylalanine ethyl ester. A_2 has no action against the acylated substrate, ATEE. Neither the A_1-type esterase nor the A_2 esterase are inhibited by AIn.

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steel cup, frozen at —25°, and after thawing, centrifuged at 28,000 × *g* for 80 minutes. The slightly hazy supernatant layer was removed from the small pellet to give Fraction MP-1. This fraction was kept in storage at —25°.

Table I summarizes the results of this fractionation. The proteolytic activity of Fraction MP-1, purified 100-fold, was the same whether exposed to *activation* or *inactivation conditions* prior to addition to the casein substrate. Thus, the activity in this fraction satisfied the first requirement for a Proteinase C preparation separated from its inhibitor. The refractoriness of the activity in Fraction MP-1 to *activation* and *inactivation* conditions was established over a wide range of enzyme concentration (Fig. 1). However, since the yield of activity in this fraction was only 30 %, and since approximately this percentage of the total activity in an initial extract (Fraction E-1.6) was unaffected by exposure to *activation* or *inactivation conditions*[1], it was clearly possible that Fraction MP-1 might not represent a purified Proteinase C preparation. A decisive clarification of this point will be presented later.

Both A_1 and Proteinase A were present in Fraction MP-1 but their combined activity represented only 9 % of the total initial esterase activity contained in the E-1.6 extract (Table I). The SAL-0.8 fraction, obtained in Step III of the fractionation scheme, contained 63 % of the total esterase activity, comprised of both A_1 and Proteinase A, and its separation from the bulk of the proteolytic activity was evident by the high esterase to proteinase ratio of 84 (compare with Fraction SAL-3.2, Table I). Thus, Step III, in which the EDP-1 fraction was adsorbed onto alumina gel at ionic strength 0.8, selectively left the greater portion of A_1 and Proteinase A in solution, whereas the major amount of the proteolytic activity remained with the gel phase.

TABLE I

PURIFICATION OF PROTEINASE AND ESTERASE ACTIVITY

Fraction	*Volume** (ml)	*Total Protein N* (mg)	*Proteinase assays***			*Esterase assays****			$\frac{[k_0]_T^{ATEE}}{[DP]_T^{cas.}}$
			$[DP]_T^{cas.}$ × 10^3	$[DP]_N^{cas.}$ × 10^3	*Per cent yield*	$[k_0]_T^{ATEE}$	$[k_0]_N^{ATEE}$	*Per cent yield*	
E-1.6	2400	1800	77,000	43		1580 (575)	0.88		20.5
EDP-1	145	122	72,000 (24,000)	590	94	1180 (230)	9.7	75	16.4
SAL-0.8	470	66.0	11,750 (7,500)	178	15	990 (188)	15.0	63	84.0
SAL-3.2	75	18.7	38,000	2000	49	127	6.8	8.0	3.3
MP-1	25	5.2	23,000 (23,000)	4400	30	142 (22)	27.3	9.0	6.2

* All volumes were corrected for the amounts withdrawn for assay and protein nitrogen determinations.

** $[DP]_T^{cas.}$, total proteinase units; $[DP]_N^{cas.}$ specific activity per mg protein nitrogen. Activity determinations were obtained using *activation conditions* except those values in parentheses which were obtained using *inactivation conditions* (see MATERIALS AND METHODS). The ionic strength of all enzyme-substrate reaction solutions was 0.80.

*** $[k_0]_T^{ATEE}$, total esterase units; $[k_0]_N^{ATEE}$, specific activity per mg protein nitrogen. Values in parentheses were obtained in the presence of a sufficient amount of the AIn inhibitor preparation to produce maximal inhibition of ATEE hydrolysis and thus are a measure of the total A_1 enzyme units[2]. $[k_0]_T^{ATEE}$ minus total A_1 units equal units of Proteinase A[2].

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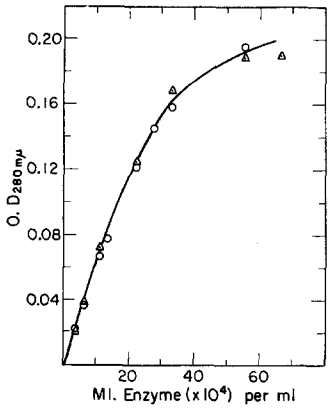

Fig. 1. Proteolytic activity versus enzyme concentration. The enzyme source was Fraction MP-1 and contained $800 \cdot 10^{-8}$ proteinase units per ml when assayed at ionic strength 0.60 by either ○, *inactivation conditions* or △, *activation conditions*.

Preparation of an inhibitor fraction

Initial experiments to prepare a fraction containing an inhibitor toward the proteinase activity of Fraction MP-1 by trichloroacetic acid precipitation or differential heat inactivation of E-1.6 extracts were only partially successful. Likewise, attempts to desorb inhibitory material from the alumina gel residue after completion of Step IV of the fractionation procedure resulted in failure. However, success in the preparation of an inhibitor fraction was accomplished by the preferential destruction of all proteolytic activity in a Step II EDP-1 fraction by exposure to pH 3.0. The inhibitor preparation was prepared at 1° as follows: Fraction EDP-1 was adjusted to pH 3.0 and ionic strength 0.48. After 180 minutes, 0.05 *N* NaOH was added to a pH of 8.5 and the ionic strength adjusted to 0.40. At high concentrations, this material did not hydrolyze either ATEE or casein.

Inhibition of proteolytic activity

In all subsequent experiments, Fraction MP-1 has been used as the enzyme source.

Reversibility of inhibition

Table II offers proof that the acid-inactivated EDP-1 preparation described above can reversibly inhibit the proteolytic activity of Fraction MP-1. Incubation with the enzyme solution at 1° for 3.0 hours at ionic strength 0.60 and pH 7.5 (*inactivation conditions*) before assay at the same ionic strength resulted in a decrease of activity which could be readily reversed by exposure to ionic strength 1.6. These results conform to those that have been reported for the ionic strength-dependent, reversible inactivation of Proteinase C in an initial skin extract which was postulated to occur by association of this enzyme with an inhibitor, CIn. It is apparent then, in conjunction with other evidence reported below, that Fraction MP-1 represents a purified Proteinase C preparation and that the acid-inactivated EDP-1 preparation contains the CIn inhibitor. This inhibitor fraction will be referred to as the CIn preparation. The absence of CIn in Fraction MP-1 is shown by the refractoriness of the proteolytic activity of this enzyme preparation to exposures of varying ionic strength prior to assay (*cf.* Fig. 1). Thus, our original explanation for the reversible inactivation of Proteinase C in skin extracts has been confirmed by (a) the isolation of Proteinase C, free of CIn;

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TABLE II

THE INACTIVATION OF PROTEOLYTIC ACTIVITY IN FRACTION MP-1 BY THE CIn PREPARATION AND ITS REVERSIBILITY

Proteolytic activity and its inhibition was determined after exposure to *inactivation conditions*. Varying quantities of Fraction MP-1 and the CIn preparation were mixed with a phosphate–KCl buffer and incubated at 1°. Inhibition controls did not contain CIn. Conditions of *incubation*: volume, 1.48 ml; ionic strength 0.60; pH 7.5; phosphate, 0.005 *M*; time, 180 minutes. At the end of the incubation period there was rapidly added 7.52 ml 0.06 *M* phosphate buffer containing 0.47 *M* KCl (Solution A) to give a total volume of 9.0 ml with the ionic strength remaining unchanged. This solution (2.0 ml) was then added to the assay flask at 35.0° containing 1.0 ml 2% casein and 1.0 ml 0.1 *M* phosphate buffer in 0.99 *M* KCl. For assay with *activation conditions* after inactivation had occurred, 5.26 ml of Solution B (0.128 *M* phosphate buffer containing 1.55 *M* KCl) were added to duplicate incubation solutions instead of Solution A to bring the total ionic strength to 1.6 and then, immediately prior to the removal of an aliquot for assay, 2.26 ml 0.1 *M* phosphate buffer were added. The final volume, as in the first set of tubes, was 9.0 ml but the ionic strength was 1.2. As above, 2.0 ml were added to assay flasks at 35.0° containing 2.0 ml 1% casein. The final conditions for either type of activity determination were 0.05 *M* phosphate buffer, pH 7.5, ionic strength 0.60, and a casein concentration of 0.5%. All buffers used were at pH 7.5.

ml CIn per ml ($\times 10^4$)	*Activity*, determined by*		*Per cent*	
	Inactivation conditions	*Activation conditions*	*Inactivation*	*Reactivation*
0	1.13			
27.8	0.49	1.19	57	105
0	2.27			
16.7	1.00	2.05	56	91
55.6	1.00	2.46	56	108
0	4.30			
111	1.50	4.00	65	93

* [DP] $\times 10^3$ units per ml assay solution.

(b) the isolation of CIn, free of Proteinase C; and (c) the ionic strength dependence of their association and dissociation.

Effect of inhibitor concentration

The relationship between CIn concentration and the activity of Fraction MP-1 against casein is shown in Fig. 2. The curves obtained are similar to those described by NORTHROP, KUNITZ AND HERRIOTT[6] for a dissociable complex in equilibrium with free enzyme and inhibitor. At the two lower enzyme concentrations used, the quantity of the CIn preparation necessary for 50% inhibition was constant and occurred at a concentration of $12 \cdot 10^{-4}$ ml per ml. At the highest enzyme concentration used, 50% inhibition was obtained at a CIn preparation concentration of $16 \cdot 10^{-4}$ ml per ml or higher (Fig. 2). The maximal inhibition of proteolytic activity in Fraction MP-1 by the CIn prepartion was 60 to 65%.

Effect of time

The time-dependence of Proteinase C inactivation upon exposure to ionic strength 0.63 at 1° has been studied at several different pH values using an E-1.6 extract[1]. With the concentration of E-1.6 extract used, maximal association was complete in approximately 240 minutes and was independent of pH over the range 5.5 to approximately 9. This experiment was repeated by following the decrease in activity of

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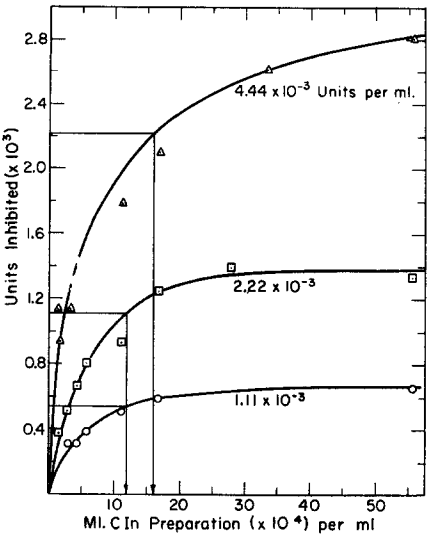

Fig. 2. Effect of the CIn inhibitor preparation on the hydrolysis of casein by Fraction MP-1. Activity determinations were performed using *inactivation conditions* and the protocol accompanying Table II was followed. The [DP] units per ml assay solution given beneath each curve represent initial proteinase units present and the abcissa values of the rectangular plots indicate the CIn concentration required for 50 per cent inhibition.

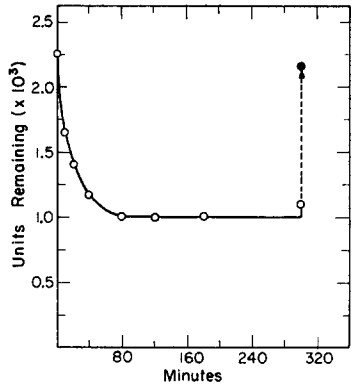

Fig. 3. The time-dependence of the Proteinase C-CIn interaction at pH 7.5, ionic strength 0.60, and 1°. Protocol as described in Table II. Assays according to *inactivation conditions* except for the point (●), which was obtained by using *activation conditions*. Concentration of CIn, $16.7 \cdot 10^{-4}$ ml per ml; enzyme concentration, $27.8 \cdot 10^{-4}$ ml of Fraction MP-1 per ml ($2.25 \cdot 10^{-3}$ [DP] units)

Fraction MP-1 upon exposure to ionic strength 0.6 at pH 7.5 and 1° in the presence of the CIn preparation. With the single concentration of enzyme and inhibitor used, the extent of inactivation approached an asymptotic limit in 80 minutes and, as previously shown (*cf.* Table II) could be reversed by exposure to ionic strength 1.6 (Fig. 3).

Properties of the Proteinase C preparation

Effect of salt concentration

Previous results have demonstrated that the rate of casein digestion at pH 7.5 by fully activated Proteinase C was dependent upon ionic strength[1]. An E-1.6 extract (*cf.* Step I, fractionation procedure) was used for such experiments and a plot of activity versus the ionic strength of assay (contributed by 0.05 *M* phosphate plus added KCl) gave a bell-shaped curve with maximum activity at ionic strength 0.6[1]. Similar results have been obtained with Fraction MP-1 using phosphate–KCl and phosphate–NaBr buffers (Fig. 4, A) and provide additional evidence for the presence of Proteinase C in this fraction.

The effect of various salts upon activity was also studied in the absence of buffer. All salts tested yielded bell-shaped activity curves similar to those in Fig. 4, A, but surprisingly, the maximum proteolytic activity in the presence of a particular salt was found to be a function of the magnitude of the ionic strength optimum (Fig. 4, B). In the presence of KCl, the ionic strength optimum was 1.0 and the activity of Fraction

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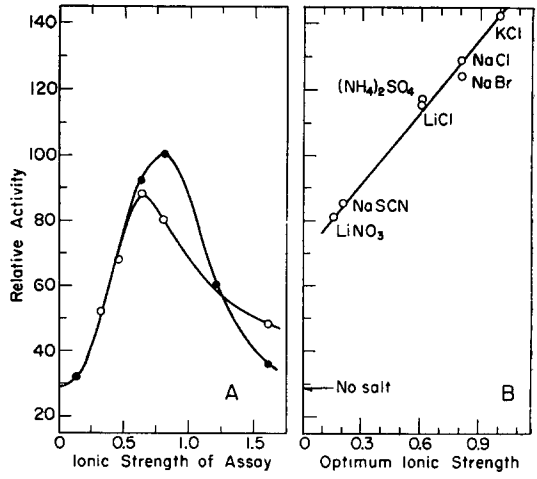

Fig. 4. A. The effect of ionic strength upon the hydrolysis of casein by Fraction MP-1. Assays were done at pH 7.5 in the presence of 0.05 *M* phosphate and added KCl (●) or NaBr (○). B. The relationship between the optimum ionic strength and the activity of Fraction MP-1. In the absence of buffer, variations in proteolytic activity were determined at pH 7.5 in the presence of various ionic strength contributors to give results similar to those shown in Fig. 4, A. The maximal activity in the presence of a particular salt was then plotted against the ionic strength at which it occurred, i.e., the ionic strength optimum. All activities are relative to that obtained in phosphate-KCl at ionic strength 0.8. The enzyme concentration was $16.7 \cdot 10^{-4}$ ml per ml. Reagent grade, C.P. salts were used throughout.

MP-1 was increased 5-fold relative to the activity at zero ionic strength. The reason for such a relationship, apparently relating the efficiency of enzyme protein to the optimal concentration of a particular salt, is not obvious. Clearly, this effect is not attributable solely to ionic strength, since ionic species differences are apparent, although it is noteworthy that the point for the uni-divalent salt, $(NH_4)_2SO_4$, is not divergent from results obtained with uni-univalent salts.

Comparison of the relative activity of Fraction MP-1 at the ionic strength optimum in the presence of phosphate-KCl (Fig. 4, A) with that in the presence of KCl alone (Fig. 4, B) shows that phosphate produces some inhibition.

The maximal activity of Fraction MP-1 was much lower in the presence of $LiNO_3$ than it was in the presence of KCl. It was thought that such a difference might be due to the choice of pH 7.5 for the measurement of casein digestion. However, in the presence of either 0.2 *M* $LiNO_3$ or 1.0 *M* KCl, the rate of casein hydrolysis by Fraction MP-1 was optimal at approximately pH 7.5. Thus the lowered activity of this fraction in $LiNO_3$ is not due to the existence of a pH optimum removed from 7.5.

Results similar to those discussed here have been reported for the effect of ionic strength upon the acid DNase activity of rat liver[7]. At pH 5, the activity increased to a maximum at ionic strength 0.15 to 0.2 and then decreased to essentially zero above ionic strength 0.8.

The effect of possible activators or inhibitors

The effect of various materials upon the proteolytic activity of Fraction MP-1 was studied by pre-incubation with the enzyme at 1° before dilution with buffer and subsequent addition to the assay flask. The conditions of incubation were identical to those used to demonstrate the inhibition of Proteinase C by the CIn inhibitor (*cf.* protocol, Table II). In all cases discussed below, the enzyme was present at a concentration of $2.0 \cdot 10^{-3}$ [DP] units per ml assay solution.

Neither soybean inhibitor, ovomucoid, lima bean inhibitor, nor the bovine plasma trypsin inhibitor produced inhibition. The highest concentration of these various inhibitors tested was sufficient to inhibit completely the activity of 6 to 7 μg crystalline

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trypsin* per ml assay solution. Trypsin activity and its inhibition were measured by the same procedure. The concentration of trypsin was calculated from the optical factor (0.695) given by GREEN AND NEURATH[8].

The AIn inhibitor, capable of completely inhibiting the activity of Proteinase A[2], had essentially no effect upon the activity of Fraction MP-1 at a concentration of 0.05 ml per ml. Cysteine at a concentration of $5 \cdot 10^{-4} M$ produced 30 % inhibition and when increased to $5 \cdot 10^{-3} M$, caused a 65 % drop in activity. Glutathione, at essentially the same concentrations, produced no effect. *p*-Chloromercuribenzoate, at concentrations ranging from 3 to $30 \cdot 10^{-5} M$ was likewise ineffectual but $HgCl_2$ ($5 \cdot 10^{-4} M$) produced 85 % inhibition. The inhibition with $5 \cdot 10^{-3} M$ $ZnCl_2$ was much less and amounted to approximately 35 %.

The results with diisopropyl phosphorofluoridate were somewhat erratic but over the concentration range tested, $0.3 \cdot 10^{-4}$ to $15 \cdot 10^{-4} M$, the degree of inhibition varied from zero to 25 %.

Substrate specificity studies

More than 45 different synthetic compounds have been tested for susceptibility to hydrolysis by the purified Proteinase C preparation. The following representative compounds were *not* hydrolyzed: carbobenzoxy-glycylglycinamide, benzoyl-L-tyrosinamide, benzoyl-L-argininamide, benzoyl-glycinamide, acetyl-glycine hydrazide, carbobenzoxy-L-leucylglycine methyl ester, carbobenzoxy-L-glutamylglycine methyl ester, carbobenzoxy-hydroxy-L-proline methyl ester, carbobenzoxy-L-tyrosylglycylglycine methyl ester, carbobenzoxy-glycyl-L-leucylglycine methyl ester, tosyl-L-arginine methyl ester, carbobenzoxy-hydroxy-L-prolyl-L-aspartic acid, carbobenzoxy-glycyl-L-phenylalanine, carbobenzoxy-L-glutamyl-L-phenylalanine, phthalyl-glycyl-L-aspartic acid, phthalyl-glycyl-L-glutamic acid, glycyl-L-phenylalaninamide, L-leucinamide, L-histidine methyl ester, L-lysine methyl ester, hydroxy-L-proline methyl ester, DL-alanylglycylglycine, DL-alanyl-DL-asparagine, DL-alanyl-DL-methionine, glycyl-L-tyrosine, and glutathione.

In contrast, the substrates acetyl-L-tryptophan ethyl ester, N-benzoyl-L-tyrosine ethyl ester, carbobenzoxy-hydroxy-L-prolyl-L-phenylalanine ethyl ester, ATEE, and TEE were hydrolyzed. It has been demonstrated in a previous paper[2] that these substrates are not hydrolyzed by Proteinase C but are hydrolyzed by Proteinase A and, with the exception of TEE, by the A_1 enzyme. Further confirmation of this point was indicated by the failure of the CIn preparation to inhibit the hydrolysis of ATEE.

Some properties of the CIn inhibitor

Exhaustive dialysis of the CIn inhibitor preparation against ion-exchanged water at 1° resulted in the complete precipitation of CIn in the dialysis casing. At the present state of purification therefore, CIn is not diffusible through cellophane.

CIn was not destroyed by heating at 70° for 10 minutes in $0.40 M$ KCl.

CIn will withstand a 3 hour exposure to pH 10.3 at 1° since a preparation of this inhibitor was made from Fraction EDP-1 under these conditions. Proteolytic activity was absent and the potency was apparently equivalent to the CIn preparation made at an acid pH.

* Worthington Biochemical Corporation, Freehold, New Jersey, (U.S.A.).

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DISCUSSION

From a consideration of the experimental results presented in this paper one can conclude that the ionic strength-dependent, reversible inactivation of Proteinase C in an initial skin extract occurs by association with an inhibitor, CIn, and that the reversal of inactivation is due to Proteinase C–CIn dissociation. This would also obtain in any other enzyme fraction containing both Proteinase C and CIn. This conclusion was made possible by the isolation of both Proteinase C and CIn, each free of the other, and by a study of their interaction. The evidence that Proteinase C, rather than non-specific proteolytic activity, had been purified and that it had been freed of the CIn inhibitor can be summarized as follows: (a) the activity was unaffected by the ionic strength environment prior to assay; (b) its hydrolysis of casein was dependent upon the ionic strength of the reaction solution; (c) its interaction with the CIn inhibitor preparation was ionic strength-dependent and reversible; and (d) its inactivation in the presence of the CIn inhibitor was time-dependent whereas the reactivation process was essentially instantaneous. Items (c) and (d) also provide evidence that an inhibitor with the requisite characteristics for the postulated CIn inhibitor[1] has been prepared.

In previous work[1], the question arose as to whether Proteinase C–CIn interaction occurred in the presence of casein when a skin extract at ionic strength 1.6, and hence containing dissociated Proteinase C and CIn, was added to the substrate. The conditions of the assay solution were such as to otherwise facilitate rapid association (ionic strength, 0.6 to 0.8; pH 7.5; temperature, 35.0°). At that time, an unequivocal answer to this question could not be obtained since the separation of Proteinase C and CIn had yet to be achieved. However, the results of inhibition experiments described in this paper, using the Proteinase C and CIn preparations argue against such an occurrence. The exposure of CIn-inhibited Proteinase C to *activation conditions* prior to assay completely reversed the inhibition and yielded activity comparable to that obtained in the absence of CIn (*cf.* Table II). Thus, the presence of 0.5 % casein prevents the association of CIn with Proteinase C and does not reverse such association once it has occurred.

The magnitude of the Proteinase C contribution to the total proteolytic activity of Fraction MP-1 is uncertain. The maximum inhibition of activity by CIn approached an asymptotic limit at 60 to 65 % inhibition, and by definition[1], at least this much of the total activity is due to Proteinase C. However, the following observations have a bearing upon this point. The activity of Fraction MP-1 is unstable upon storage at —20°, a 40 % loss occurring in 6 months. Yet, even in those cases where the total initial activity was decreased by 50% or more, the inhibition of remaining activity by the CIn preparation never exceeded 60 to 70%. The shape of these inhibition curves were identical to those illustrated in Fig. 2 which were obtained with a fresh MP-1 preparation. Storage-inactivated MP-1 fractions also showed an activity dependence upon the ionic strength of assay identical to that shown in Fig. 4, A. These results would suggest that Proteinase C contributed more to the total activity of Fraction MP-1 than the inhibition experiments with CIn would indicate, since a storage-inactivated MP-1 preparation resembles a fresh MP-1 fraction in its reactivity toward the CIn preparation and in its relative activity at different ionic strengths of assay. By analogy, WU AND LASKOWSKI[9] have shown that colostrum inhibitor does not completely inhibit the activity of α-chymotrypsin, *e.g.*, only 5 μg of the 9.5 μg α-chymotrypsin present were inhibited at the asymptotic limit of inhibition.

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A differentiation of the A_1 enzyme and Proteinase A from Proteinase C was obtained by heating at 50°[2]. The latter enzyme was unstable to such treatment. Further confirmation of the non-identity of Proteinase C with either of the above two enzymes can now be offered. The pertinent distinctions are as follows: (a) A_1 plus Proteinase A can be partially separated from Proteinase C; (b) the CIn inhibitor has no effect upon A_1 or Proteinase A; (c) the Proteinase A inhibitor, AIn, does not inhibit the activity of the Proteinase C preparation; (d) soybean inhibitor, lima bean inhibitor, bovine plasma trypsin inhibitor, and diisopropyl phosphorofluoridate are unable to inhibit Proteinase C whereas both A_1 and Proteinase A are completely inhibited by such materials[2]; and (e) the proteolytic activity of the Proteinase C preparation was decreased at —20° without an attendant decrease in the activity of either A_1 or Proteinase A.

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SUMMARY

1. The purification of Proteinase C and the separation from its inhibitor (CIn), the A_1 enzyme, and Proteinase A has been achieved.

2. The CIn inhibitor has been obtained free of proteolytic activity, the A_1 enzyme, and Proteinase A.

3. Studies on the interaction between purified Proteinase C and CIn lead to the conclusion that the activity of this enzyme in skin extracts is dependent upon its degree of association with CIn; the extent of such association being primarily a function of ionic strength.

4. The properties of purified Proteinase C have been studied.

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