SUMMARY

Firefly luciferin ($C_{13}H_{12}N_2S_2O_3$) reacts with ATP to form active luciferin (apparently adenyl-luciferin) and pyrophosphate. The oxidation of active luciferin leads to light emission and adenyl-oxyluciferin, the latter compound eventually decomposes into adenylic acid and oxyluciferin ($C_{13}H_{10}N_2S_2O_3$). Oxyluciferin is a potent inhibitor of the light reaction and once it has reacted with ATP and luciferase, the latter is incapable of catalyzing the oxidation of luciferin. Coenzyme A stimulates light emission by removing oxyluciferin from the enzyme surface. The evidence indicates that oxyluciferyl-CoA is formed, which can react non-enzymically with cysteine, glutathione or hydroxylamine to form the corresponding oxyluciferyl derivatives. Chromatographic, isotopic and fluorometric data are presented to support the above conclusions. Oxyluciferyl-CoA in the presence of luciferase can be split by adenylic acid and when excess pyrophosphate is added ATP and free oxyluciferin are formed. The incorporation of ^{14}C-adenylic acid into ATP depends upon the presence of CoA in the reaction mixture. The importance of these various reactions for light emission and electron transport is discussed.

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THE PROTEOLYTIC ENZYME SYSTEM OF SKIN

IV. THE PURIFICATION OF PROTEINASE A*

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Aromatic amino acid esters and their substituted derivatives can be hydrolyzed by at least three enzymes, *viz.*, Proteinase A, the A_1 enzyme and the A_2 enzyme, present in extracts of rat skin acetone powder[1]. Both the A_1 enzyme and Proteinase A attack acylated amino acid esters and acylated and non-acylated dipeptide esters in which

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References p. 538.

the carboxyl group of an aromatic amino acid is involved in ester linkage[2]. Differentiating substrates for these two enzymes are non-acylated aromatic amino acid esters, *e.g.*, L-tyrosine ethyl ester (TEE) and L-phenylalanine ethyl ester (PEE), which are hydrolyzed by Proteinase A but not by the A_1 enzyme[2]. These two substrates are also hydrolyzed by the A_2 enzyme but both A_1 and Proteinase A can be distinguished from A_2 by the inability of the latter enzyme to split substituted aromatic amino acid esters, *e.g.*, N-acetyl-L-tyrosine ethyl ester (ATEE). A further means for their differentiation depends upon the stoichiometric inhibition of only Proteinase A by an inhibitor, AIn, isolated from sheep blood[2].

This paper will describe the purification of Proteinase A and its separation from the A_1 enzyme (partial) and the A_2 enzyme (complete). It will also be shown that Proteinase A, has been separated from Proteinase C[3,4]. This latter enzyme is the major contributor in skin extracts to the proteolytic activity against casein and its activity is unaffected by the Proteinase A blood inhibitor, AIn[4].

EXPERIMENTAL

Rat skin acetone powder[3], ATEE[2], TEE[2], and AIn[2] were previously reported preparations. Protein nitrogen was estimated as before[2].

Measurement of activity

Previously described methods for the determination of proteolytic[3] and esterase[2] activity have been used. Progress in the purification of Proteinase A was followed by the use of ATEE and TEE as substrates. The assay of Proteinase A in the presence of the A_1 enzyme was conducted with ATEE as the substrate at pH 8.0 and permitted a concomitant estimate of A_1 activity. The total hydrolysis rate due to both enzymes was first determined and then re-determined in the presence of sufficient AIn to completely inhibit the activity of Proteinase A. The rate measured with AIn present was used to calculate units of A_1 activity and when subtracted from the total esterase activity, determined in the absence of AIn, gave an estimate of the activity of Proteinase A. The reliability of such rate measurements to determine Proteinase A activity in the presence of another enzyme attacking the same substrate was checked by using the Proteinase A-specific substrate, TEE. The hydrolysis of this ester (and of PEE) was measured at the optimum pH of 6.5[2].

A value of unity for the apparent zero-order reaction constant, k_0, defined as the μmoles substrate hydrolyzed per minute per ml enzyme, equals one esterase unit and the symbol, $[k_0]_T^S$, represents total esterase units using an ester, S, as the substrate. The designation, $[E]_T^S$ and $[E]_N^S$ will be used to represent the total units and the specific activity per mg protein nitrogen, respectively, of a particular enzyme $[E]$, viz., A, A_1 or A_2, where the substrate, S, can be either an ester or casein.

Fractionation procedure

Although the first three steps of the fractionation have been given in detail elsewhere[4], they will be briefly described below to maintain continuity of presentation. All operations were performed at 1°.

Step I. Extraction. Treatment of 550 g rat-skin acetone powder with 1.6 *M* KCl gave 7.03 l of initial extract, Fraction E-1.6.

Step II. Dialysis. Fraction E-1.6 was dialyzed against ion-exchanged water to yield a precipitate which was suspended in 0.60 *M* KCl, Fraction EDP-1. This fraction contains Proteinases A and C and the A_1 enzyme and is free of A_2. The aqueous phase from the dialysis step, Fraction S-1, is free of Proteinases A and C and contains the A_2 enzyme and A_1-type activity*.

Step III. Treatment of Fraction EDP-1 with alumina gel. Fraction EDP-1 was mixed with alumina gel[4] at ionic strength 0.8, the suspension centrifuged, and the supernatant fluid removed to give Fraction SAL-0.8. The greater portion of the Proteinase C activity remains adsorbed onto the alumina gel.

Step IV. Heat treatment of Fraction SAL-0.8. One volume of fraction SAL-0.8 was diluted with 0.34 volume 4.0 *M* KCl to give ionic strength 1.6 and heated in a bath at 50° for 60 minutes

* Results to be published.

References p. 538.

to destroy contaminating Proteinase C activity. After cooling to 1°, the solution was placed in $1^5/_8$ inch expanded cellophane casings (600 ml per casing) and each sack dialyzed for 24 h against approximately three 12-volume changes of ion-exchanged water. The material was then centrifuged at 3500 *g* for 60 min, the supernatant fluid discarded, and the precipitate suspended in 0.60 *M* KCl to a final volume equivalent to 0.09 ml/ml of solution dialyzed, Fraction HDP-0.6.

Step V. Treatment of Fraction HDP-0.6 with alumina gel. Equal volumes of alumina gel and Fraction HDP-0.6 were mixed to give ionic strength 0.30 and after standing for 10 min, the suspension of fibrous material was centrifuged at 3500 *g* for 40 min. The supernatant solution was discarded and the precipitate eluted with 0.19 ml 0.1 *M* phosphate buffer containing 1.34 *M* KCl, pH 7.5 (ionic strength 1.6) per ml Fraction HDP-0.6 used. The eluate was collected by centrifugation at 25,000 *g* for 30 min and the residue re-extracted with the same solvent (0.13 ml/ml Fraction HDP-0.6 used). The two eluates were combined, the ionic strength reduced to 0.40 by dilution with water, and after 30 min, the suspension was centrifuged at 3500 *g* for 60 min. The hazy supernatant solution was clarified by filtration through Whatman No. 12 paper (24 cm) to give Fraction SA-I.

Step VI. Precipitation of Fraction SA-I at pH 5.0. Fraction SA-I was adjusted to pH 5.0 with 0.025 *N* HCl, and ionic strength 0.20. The requisite amount of HCl was determined from the results of a titration curve. After one hour, the suspension was centrifuged at 3500 *g* for 130 min, the supernatant fluid discarded, and the precipitate dissolved in 0.40 *M* KCl (0.24 ml per ml Fraction SA-I used) plus a few drops of 0.1 *N* NaOH. This material was then stored at —20° for several weeks, thawed, and centrifuged at 28,000 *g* to give a clear supernatant solution, Fraction PA-II.

Step VII. Dialysis. Fraction PA-II was dialyzed for 24 h against two 160-volume changes of ion-exchanged water and the suspension obtained centrifuged at 28,000 *g* for 40 min. The resulting precipitate was mixed with 0.40 *M* KCl (0.20 ml/ml Fraction PA-II used) and the insoluble material removed by centrifugation at 28,000 *g* for 40 min to give the almost water-clear supernatant PA-III fraction*.

RESULTS AND DISCUSSION

Table I summarizes the results of esterase-activity measurements on all fractions obtained during the purification of Proteinase A. Fraction PA-III, with an $[A]_N^{ATEE}$ of 500, represents an 850-fold purified preparation of this enzyme in approximately 40% yield. The same purification factor and yield can also be calculated from the results of assays using TEE as the substrate since the ratio of $[A]_N^{ATEE}/[A]_N^{TEE}$ was constant at each stage of purification. The significance of this ratio invariance is two-fold. Firstly, it provides evidence that the activity of Proteinase A can be determined as the difference in the rate of total ATEE hydrolysis, due to both Proteinase A and the A_1 enzyme, to that measured in the presence of the Proteinase A inhibitor, AIn, *i.e.*, wherein only the A_1 enzyme is operative. Secondly, it can be considered proof that the designation, Proteinase A, applies to only a single enzyme moiety since it would be extremely fortuitous that two AIn preparation-sensitive enzymes capable of attacking ATEE and TEE or alternatively, the one attacking ATEE and the other TEE, would be carried throughout the fractionation procedure in constant proportion to an 850-fold degree of purification. Obviously, these points lean strongly upon the implied assumption that TEE can be used to determine the activity of Proteinase A by direct assay. However, the use of TEE as a specific Proteinase A substrate is complicated by the fact that TEE can also be hydrolyzed by the A_2 enzyme. But fortunately, as stated earlier, A_2 activity is separated from Proteinase A in Step II of the fractionation procedure and the material obtained from such a step, Fraction EDP-1, is free of enzyme A_2. The criterion of A_2 removal from a Proteinase A preparation is dependent upon realizing the complete abolish-

* It is now known that Fraction PA-II can be concentrated with no loss of Proteinase A activity by lyophilization.

References p. 538.

TABLE I

THE PURIFICATION OF PROTEINASE A

Fraction	*Volume* (ml)*	*mg Protein N*	$[A_1]_T^{ATEE}$**	$[A]_T^{ATEE}$***	$[A]_T^{TEE}$	$[A_1]_N^{ATEE}$	$[A]_N^{ATEE}$	$[A]_N^{TEE}$	$\frac{[A]_N^{ATEE}}{[A_1]_N^{ATEE}}$	$\frac{[A]_N^{ATEE}}{[A]_N^{TEE}}$
E-1.6	7030	4360	1480	2550	291§	0.34	0.59	0.067	1.7	8.8
EDP-1	423	305	782	2600	262	2.6	8.5	0.86	3.3	9.9§§
SAL-0.8	1395	181	670	2300	278	3.7	13	1.5	3.5	8.7
HDP-0.6	170	46	570	1500	174	12	33	3.8	2.8	8.7
SA-I	217	—	330	1280	—	—	—	—	—	—
PA-II	53	3.8	355	1290	—	94	340	—	3.6	—
PA-III	11.3	1.9	190	950	109	100	500	57	5.0	8.8

* Corrected for amounts withdrawn for assay.

** Determined in the presence of sufficient AIn to inhibit completely Proteinase A.

*** Equal to $[k_0]_T^{ATEE} - [A_1]_T^{ATEE}$.

§ A calculated value (see APPENDIX). $[k_0]_T^{TEE}$ for Fraction E-1.6 was 815.

§§ Three other EDP-1 fractions gave values of 8.6, 9.2, and 8.9.

ment of TEE (or PEE) hydrolysis in the presence of AIn. Fraction EDP-1 and subsequent fractions obtained during the course of purification satisfied this requirement.

PEE, although a markedly less susceptible substrate for Proteinase A than TEE, has also been used to confirm the degree of purification of this enzyme and the results for Fraction E-1.6 ($[A]_N^{PEE}$, 0.010) and for Fraction PA-III ($[A]_N^{PEE}$, 9.0), which yield a purification factor of 900-fold, confirm the purification factor of 850-fold obtained with ATEE and TEE as substrates. The results with PEE lend further credence that Proteinase A is a single enzyme.

The appendix to this paper will describe methods for the differential assay of Proteinase A and A_2 in initial skin extracts using ATEE, TEE, and PEE as substrates.

The next to the last column in Table I shows that the $[A]_N^{ATEE}/[A_1]_N^{ATEE}$ ratio was increased 3-fold during the purification of Proteinase A. Only 13% of the initial A_1 activity was present in Fraction PA-III.

The esterase activity of Fraction PA-III was stable to storage at —20°. However, in the purified state, both Proteinase A and the A_1 enzyme were unstable to dialysis against water as indicated by the loss of activity in Steps IV and VII of the fractionation procedure (Table I).

Evidence for the absence of Proteinase C in Fraction PA-III

Proteinase C and the Proteinase C inhibitor, CIn, have been discussed in a preceding paper[4] and it was shown that both components are present in Fraction SAL-0.8 (Step III, fractionation procedure). Since it was desirable to remove this enzyme from preparations containing Proteinase A, the activity of Proteinase C was selectively destroyed by exposure of Fraction SAL-0.8, in solution at ionic strength 1.6, to 50° as described in Step IV of the fractionation procedure. With this treatment, the total proteolytic activity against casein was decreased to an asymptotic limit in approximately 20 min, whereas the esterase activity against ATEE, due to A_1 and Proteinase A was unharmed even after an 180-min exposure (Fig. 1). The heat-stable proteolytic activity remaining was unaffected by exposure to either *activation* or *inactivation conditions*[2,3,4] for Proteinase C and was not inhibited by the CIn preparation and thus indicated the complete destruction of Proteinase C. As an added

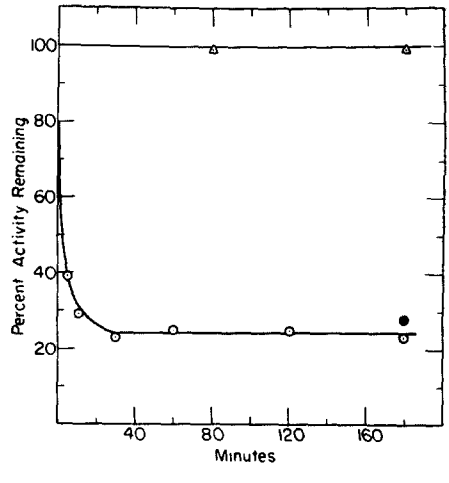

Fig. 1. The destruction of Proteinase C by heat. The esterase (△) and proteinase (○) activity of Fraction SAL-0.8 was determined after various periods of heating at 50° using ATEE and casein as substrates. The ionic strength of the enzyme preparation was 1.6 (*cf.* Step IV, fractionation procedure). Proteinase activity was determined after exposure to *activation conditions* (○) and *inactivation conditions* (●).

References p. 538.

check, the proteolytic activity of Fraction PA-III against casein was also found to be refractory to the CIn preparation, whereas it was almost completely inhibited by the AIn preparation. That which was not inhibitable by the latter inhibitor preparation, amounting to less than 10% of the total activity, might possibly reflect the ability of the A_1 enzyme to hydrolyze casein. This, however, can only be entertained as speculation since evidence is not yet available to decide whether the A_1 enzyme can hydrolyze a protein substrate.

Correlation of the proteolytic and esterase activity of Proteinase A with purification

In a previous paper[2], it was tentatively proposed that Proteinase A was an "enzyme whose esterase activity against acylated and non-acylated amino acid esters and dipeptide esters as well as its proteolytic activity against casein can be inhibited by the AIn preparation". This definition was based upon the following facts: (a) the AIn-inhibitable esterase activity paralleled the extraction of AIn-inhibitable proteolytic activity from skin acetone powder as a function of ionic strength; (b) both types of activity were found in Fraction EDP-1 (*cf.* Step II of the fractionation procedure); and (c) their stability to heating at 50° was comparable. Further evidence for the above definition can now be offered. The AIn-inhibitable esterase units and the AIn-inhibitable proteinase units were measured in two fractions, HDP-0.6 and PA-III. Both of these fractions were free of Proteinase C and the specific activity, $[A]_N^{ATEE}$, differed by almost 12-fold. The AIn-inhibitable esterase to proteinase ratio was constant (last column, Table II).

TABLE II

THE CORRELATION OF AIn-INHIBITABLE ESTERASE AND PROTEINASE ACTIVITY WITH PURIFICATION OF PROTEINASE A

Fraction	$[DP]_{ml}^{cas.} \times 10^3$	$[A]_{ml}^{cas.} \times 10^3$	$[A]_N^{cas.} \times 10^3$	$[A]_N^{ATEE}$	$\frac{[A]_N^{ATEE}}{[A]_N^{cas.}}$
HDP-0.6*	51**(5.4)***	46§	184	43	233
PA-III	415** (44)***	371§	2180	500	230

* A different preparation than that described in the text.
** Total proteinase units per ml of fraction.
*** Total proteinase units per ml of fraction with excess AIn preparation present (0.015 ml/ml) in the assay solution containing $1.13 \cdot 10^{-3}$ and $1.75 \cdot 10^{-3}$ proteinase units per ml of Fraction HDP-0.6 and PA-III, respectively.
§ AIn-inhibitable proteinase units per ml of fraction.

The properties of purified Proteinase A and the kinetics of its interaction with AIn, whose further purification is now in progress, are planned to be presented in a future publication.

SUMMARY

Proteinase A, an enzyme whose esterase and proteinase activity can be completely abolished by an inhibitor in blood, has been purified 850-fold in approximately 40% yield. The purified enzyme preparation is free of Proteinase C and the A_2 enzyme, but a complete separation from A_1 activity was not achieved.

References p. 538.

APPENDIX

The differential assay of Proteinase A and the A_2 enzyme

We presently plan to undertake studies of the skin proteolytic enzyme system which will necessitate the quantitation of Proteinase A and A_1 and A_2 enzyme activities in an initial skin extract before and after various insults to an organism. The assay of Proteinase A in the presence of A_1 with ATEE as substrate and employing AIn as a selective inhibitor for the former enzyme has been adequately described in this and a preceding paper[2]. However, an analogous differential assay for Proteinase A and the A_2 enzyme with TEE as substrate cannot be used since the AIn preparation stimulates A_2 activity in an initial skin extract[1]. This section then will describe an apparently valid means for such an assay and indicate the manner in which the value of $[A]_T^{TEE}$ and $[A]_T^{PEE}$ (*cf.* Table I and text) were calculated.

The activity of Proteinase A in Fraction E-1.6 against TEE and PEE was computed from

$$[A]_T^{TEE} = [A]_T^{ATEE} \times C$$

and

$$[A]_T^{PEE} = [A]_T^{ATEE} \times C_1$$

where $C = 0.114$ and $C_1 = 0.0171$. The symbols have the meaning indicated in the EXPERIMENTAL section. The values for C and C_1 represent the average ratio of Proteinase A activity against the substrate pairs TEE/ATEE and PEE/ATEE, respectively, and were determined with several different enzyme preparations at various stages of purification and with *A_2 enzyme activity absent.* The value for $[A]_T^{ATEE}$ was obtained in the manner indicated in Table I.

By using the experimentally determined values for the hydrolysis of TEE ($[k_0]$, 815) and PEE ($[k_0]$, 600) by Fraction E-1.6 and making the appropriate correction for the contribution of Proteinase A to the hydrolysis of these substrates (see above), one can calculate A_2 activity against TEE and PEE as follows:

$$[A_2]_T^{TEE} = [k_0]_T^{TEE} - ([A]_T^{ATEE} \times C)$$
$$= [k_0]_T^{TEE} - [A]_T^{TEE}$$
$$= 815 - 290 = 525$$

and

$$[A_2]_T^{PEE} = [k_0]_T^{PEE} - ([A]_T^{ATEE} \times C_1)$$
$$= [k_0]_T^{PEE} - [A]_T^{PEE}$$
$$= 600 - 44 = 556$$

Since preparations of the A_2 enzyme which have been obtained free of Proteinase A hydrolyze TEE and PEE at equal rates*, $[A_2]_T^{TEE}$ should equal $[A_2]_T^{PEE}$. The calculated agreement in these values is felt to be within the experimental error of the method and to provide justification for the above means of determining the activity of Proteinase A and the A_2 enzyme when these two enzymes exist in a common extract.

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