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HYPODERMIN A, A TRYPSIN-LIKE NEUTRAL PROTEINASE FROM THE INSECT *HYPODERMA LINEATUM*

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Summary

Hypodermin A, a serine proteinase from the larva *Hypoderma lineatum*, with a molecular weight of 27 000 was obtained in pure form by ion-exchange chromatography It is inhibited by diisopropyl phosphofluorate, a serine proteinase inhibitor, but not by metallo or cysteine enzyme inhibitors such as EDTA or thiol reagents. In the same way, it is fully inactivated by trypsin inhibitors, but not by specific chymotrypsin inhibitors. Its specificity, limited to carboxyl side of arginine residue in B-chain of insulin, is more complicated on other polypeptide substrates. Sequence analysis suggests structural homology with *H lineatum* collagenase as well as with other members of the trypsin family.

Introduction

The first instar larvae of the fly *Hypoderma lineatum* are parasites of domestic cattle. They migrate during 8 months through the host connective tissue, which they degrade by excreted proteolytic enzymes. The mixture of the proteolytic enzymes and degraded protein then accumulates in the digestive tract of the larvae, the presence of proteolytic and namely collagenolytic activity in this organ was demonstrated by Lienert and Throssel [1] and by Boulard [2]. Recently, Lecroisey et al. [3] characterized a homogeneous collagenase which can be obtained from the larvae in high yields. During the purification

Abbreviations BAEE, *N*-α-benzoyl-L-arginine ethyl ester, BTEE, *N*-α-benzoyl-L-tyrosine ethyl ester, TAME, *p*-tosyl-L-arginine methyl ester, BAPA, *N*-α-benzoyl-L-arginine-*p*-nitroanilide, NPGB, *p*-nitrophenyl-4-guanidino benzoate hydrochloride, TLCK, tosyl-lysyl chloromethyl ketone, TPCK, tosyl-phenylalanine chloromethyl ketone.

steps, collagenase was separated from another proteinase acting on BAEE as substrate

The simultaneous secretion of extracellular collagenase and proteinases of different specificity, complementary in their action is common in many procaryotes. In the anaerobic *Clostridium histolyticum*, the two major extracellular proteinases are a collagenase (EC 3 4 24 3) and clostripain (EC 3 4.22 8), which preferentially cleave the arginyl bonds in synthetic and protein substrates [3,24] The aerobic *Achromobacter iophagus* simultaneously produces a collagenase (EC 3.4.24 8) and several proteinases also acting on synthetic arginine substrates The simultaneous occurrence of proteinases with collagenolytic and trypsin-like specificity was also observed in the fungus *Entomophthora coronata* (EC 3.4 21 33) [4] and other fungi [5]

Currently, only a few proteinases from insects have been fully characterized as regards their active sites. A chymotrypsin-like protease from the larva of the hornet *Vespa crabra* was thoroughly studied by Jang et al. [6]. Inhibition studies have shown that cocoonase from the moth *Bombyx mori* (EC 3.4.21.4) [7] as well as the β-proteinase from the blow fly *Tenebrio molitor* (EC 3.4.21.18) [8,9] are trypsin-like proteinases. The same is true for *H lineatum* collagenase which is homologous with trypsin [25]

The observation that *H lineatum* larvae produce simultaneously with collagenase, a proteinase which acting on trypsin substrates prompted us to isolate it in pure form and to characterize some of its chemical and enzymatic properties

The present study puts forward the evidence, that hypodermin A from *H lineatum* is a serine proteinase structurally homologous with other members of the trypsin family which cleave preferentially the arginyl bond in the B-chain of insulin

Materials

Crude protease was a by-product of the purification of the collagenase from the larvae of *H lineatum* as described elsewhere [3]. Soybean trypsin inhibitor and chicken egg white purified ovomucoid were purchased from Sigma, cytochrome *c*, chymotrypsin, serum albumin and B-chain of insulin from Boehringer; carboanhydrase from Serva and bovine pancreatic trypsin inhibitor from Worthington. Parvalbumin was prepared as described by Pechère et al. [10].

N-α-Benzoyl-D-arginine ethyl ester hydrochloride, diisopropyl phosphofluoride, benzamidine and disodium EDTA were obtained from Fluka, *p*-tosyl-L-arginine methyl ester hydrochloride and *N*-α-benzoyl-D-L-arginine *p*-nitroanilide were purchased from Sigma. *p*-Nitrophenyl-L-guanidino benzoate hydrochloride, *N*-α-tosyl-L-lysyl chloromethyl ketone, *N*-α-tosylphenylalanine chloromethyl ketone were products of Merck and *N*-α-benzoyl-L-tyrosine ethyl ester was from BDH Biochemicals.

Dextran blue 2000, Sephadex G-100 were from Pharmacia and DE-32 cellulose from Whatman Biochemicals Ltd Sodium dodecyl sulfate (SDS) (Sigma) was recrystallized from ethanol.

Methods

Purification of hypodermin A The larvae of *H. lineatum* were pounded and centrifuged as previously described [3] The supernatant was passed through a

DE-32 cellulose column, equilibrated with 50 mM Tris-HCl, pH 7 5, 50 mM in $CaCl_2$. The fractions of the first peak which present an esterase activity were pooled, dialyzed against NH_4HCO_3 buffer 50 mM, pH 7.8, and lyophilized. The pure protease was obtained by a gel filtration on Sephadex G-100 in 50 mM NH_4HCO_3 buffer, pH 7.8.

Esterase and amidase activity Esterase activity was determined by measurement of the cleavage of BAEE (0.75 mM in 50 mM Tris-HCl pH 7.5, 50 mM in $CaCl_2$) at 253 nm (Zeiss PM Q III spectrophotometer) as described by Schwert and Takenaka [11]. The hydrolysis of TAME (1 mM in 40 mM Tris-HCl pH 8.1, 10 mM in $CaCl_2$) was followed at 247 nm [12]. Tryptic amidase activity was determined using BAPA as substrate (1 mM in 0.1 M Tris-HCl pH 7.8, 25 mM in $CaCl_2$), according to the method of Erlanger et al. [13]. The method of Hummel [12] with BTEE as substrate was used for chymotryptic activity. Activity was expressed in units (1 μmol substrate cleaved per min at 37° C) per mg of protein.

Active site titration The titration of the active site was carried out as described for trypsin by Chase and Shaw [14] The value was calculated from the burst of *p*-nitrophenol following the hydrolysis of NPGB.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the procedure of Ames [15], at pH 7.5 using a 5—15% gradient, with bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c* as standards. The proteins were stained with Coomassie blue R-250.

Molecular weight determination. The molecular weight of the protease was determined by the method of Andrews [16] using a Sephadex G-100 column (0.9 × 100 cm) equilibrated with 50 mM NH_4HCO_3 buffer, pH 7.8, and calibrated with pure standard proteins as for electrophoresis, and by SDS-polyacrylamide electrophoresis as described above.

Disulfide bond determination. The sensitive method of Weitzman [17] can discern between free thiol groups and disulphides. The thiol content in hypodermin A (1 mg/ml in guanidine hydrochloride, 6 M in 50 mM acetate buffer, pH 4) before and during electroreduction was determined spectrophotometrically at 412 nm, after the reaction with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid).

Amino acid analyses. Amino acid analyses were performed with a Beckman Multichrom B amino acid analyser. Samples were hydrolyzed in 5.7 N HCl at 110° C for 24, 48 and 72 h. Cysteine and methionine content was obtained after oxidation by the method of Hirs [18]. Tryptophan was determined after hydrolysis with methane sulphonic acid according to Liu and Chang [19].

Sequence analysis. Automated Edman degradation was performed in a Beckman 890 C sequencer using dimethylbenzylamine buffer [20]. The phenylthiohydantoin derivatives of the amino acids were determined by TLC [21] and by high-pressure liquid chromatography [22] on a LDC apparatus.

Temperature stability and pH optimum The thermal stability of the protease was determined by incubation of the enzyme (0.1 mg/ml in 0.1 M Tris-HCl pH 7.5, 50 mM in $CaCl_2$) at different temperatures for 10 min. The loss of esterase activity was measured with BAEE as substrate. To determine the pH optimum, esterase activity was measured with 10 mM BAEE in various

buffers (phosphate buffers: pH 3, 4.6, 5.5, 6.3, 7.5 and 8; carbonate-bicarbonate buffers: pH 8.9, 9.9 and 10.4).

Inhibition studies The effect of various trypsin inhibitors (soybean trypsin inhibitor, ovomucoid, basic pancreatic trypsin inhibition) on the activity of the protease was examined by preincubation of the enzyme and the inhibitor at various concentrations for 15 min, at 25°C, in the same buffer as for esterase activity. The residual esterase activity against BAEE containing the same concentration of inhibitor was then measured. The same method was used for inhibition by diisopropyl fluorophosphate, TLCK and TPCK, EDTA and benzamidine in excess at various incubation times and without preincubation.

Specificity studies. Hydrolysis of the B-chain of insulin and of parvalbumin (0.5 mg/ml in 50 mM Tris-HCl, 50 mM $CaCl_2$ pH 7.5) by hypodermin A (enzyme to substrate ratio. 1/100) was carried out by incubation at 37°C for 4 and 16 h The analysis of the products was effected by automatic Edman degradation.

Results

Enzyme purification, homogeneity and molecular weight. In Fig. 1 a typical chromatographic pattern of the first step of purification obtained with a DE-32

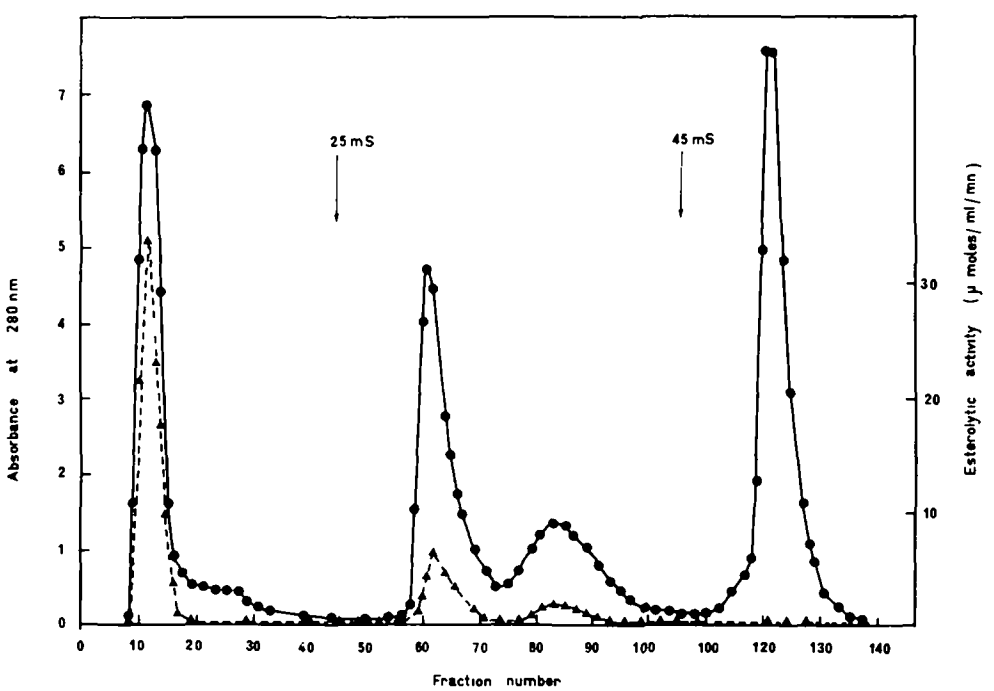

Fig 1. Chromatography of crude extract of *H lineatum* larvae on DE-32 cellulose A column (17 X 2 cm) was equilibrated with 50 mM Tris-HCl buffer pH 7.5, 50 mM in $CaCl_2$. A stepwise elution was made by subsequent application of two Tris-HCl buffers (1) 50 mM Tris-HCl buffer pH 7.5 adjusted to a conductance of 25 mS with NaCl, (2) 50 mM Tris-HCl buffer pH 7 5 adjusted to a conductance of 40 mS with NaCl The flow rate was 10 ml/h, fraction volumes 2 5 ml. All procedures were carried out at 4°C ●———●, absorbance at 280 nm, ▲ - - - - - - ▲, BAEE activity.

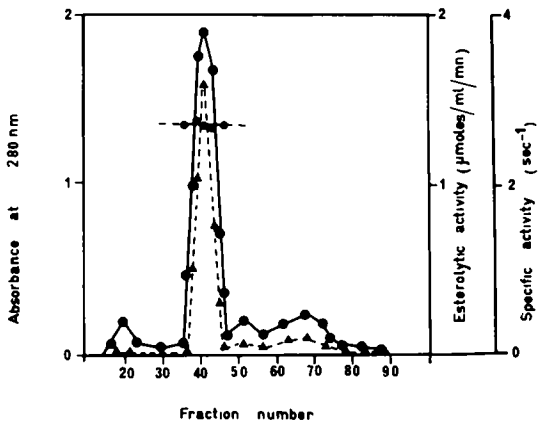

Fig. 2 Chromatography of purified *H. lineatum* proteinase on Sephadex G-100. A column (100 X 0.9 cm) was equilibrated in 50 mM NH_4HCO_3 buffer, pH 7.8. The flow rate was 10 ml/h, fraction volumes 2 5 ml ●———●, absorbance at 280 nm, ▲- - - - - - ▲, BAEE activity, ● - - - - - - ●, specific activity

cellulose column can be seen. The first peak contains the major BAEE activity and the last represents collagenolytic activity.

After lyophilization, the pooled material of the first peak was applied to a column of Sephadex G-100 (Fig. 2). Only a small amount of inactive protein separated from the second peak containing all the proteolytic activity with an homogeneous specific activity along the peak; this was pooled and lyophilized.

The yields and activities of the products of the two steps of purification are summarized in Table I. Gel electrophoresis in the presence of SDS demonstrates the homogeneity of the enzyme (Fig. 3).

The apparent molecular weight of the proteinase as estimated by means of a Sephadex G-100 column was 27 000 which agrees with the value obtained by SDS-polyacrylamide electrophoresis.

Amino acid composition and sulfhydryl groups determination. The results of the amino acid analyses are presented in Table II, in which they are compared with amino acid compositions of various serine proteinases of similar molecular weight.

Four sulhydryl groups are obtained by electroreduction of disulfide bonds and substitution by Ellman's reagent (Fig. 4).

Temperature stability and pH optimum The loss of esterase activity of hypodermin A with the increase of temperature is shown in Fig. 5A. Below

TABLE I

RECOVERY AND SPECIFIC ACTIVITY RESULTING FROM PURIFICATION STEPS OF HYPODERMIN A

Purification step	Weight (mg)	Specific activity (BAEE, U/mg)	Recovery
Crude	500	98	100
DE-32 cellulose	122	341	85
Sephadex G-100	85	468	81

TABLE II

COMPARISON OF AMINO ACID COMPOSITION OF HYPODERMIN A AND OTHER SERINE PROTEASES

Amino acid	Hypodermin A	Collagenase *H lineatum* [3]	Cocoonase *A pernyi* [31]	Cocoonase *A polyphemus* [7]	Starfish trypsin [29]	Bovine trypsin [27]	Chymotrypsin [30]	Pig elastase [26]
Half-cystine	3—4	6	4	6	8	12	10	8
Aspartic acid	22	27	26	23	31	22	22	24
Methionine	2	3	1—2	2	2	2	2	2
Threonine	6	17	16	15	15	10	22	19
Serine	22	18	23	18	18	33	27	22
Glutamic acid	19	20	15	14	20	14	15	19
Proline	10	9	13	10	13	9	9	7
Glycine	20	21	22	26	28	25	23	25
Alanine	13	11	16	16	16	14	22	17
Valine	20	19	20	19	19	12	23	27
Isoleucine	19	18	12	14	11	17	10	10
Leucine	15	14	12	15	13	10	19	18
Tyrosine	10	11	9	9	8	3	4	11
Phenylalanine	4	8	5	4	5	14	6	3
Lysine	11	6	13	12	9	14	14	3
Histidine	5	4	4	4	4	3	2	6
Arginine	11	6	6	9	4	2	3	12
Tryptophan	4	5	3	2	5	4	8	7
Number of residues	216—217	223	220—221	218	229	239	241	240

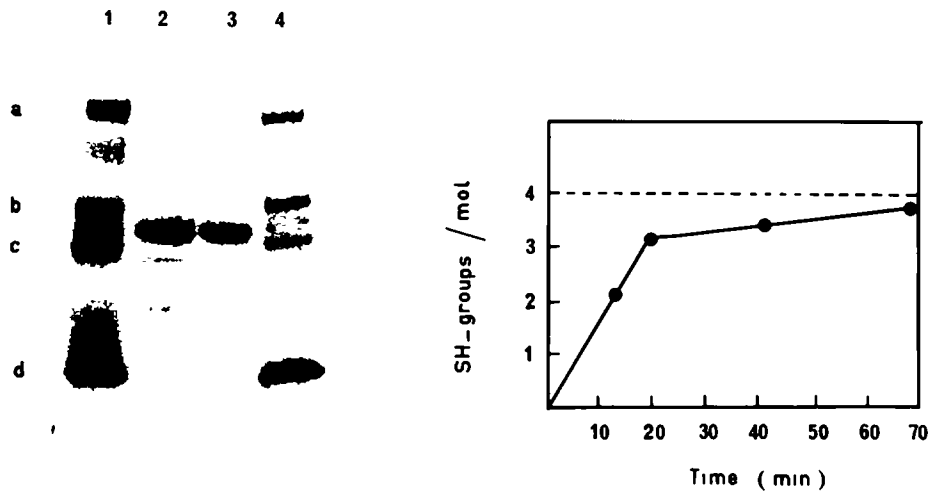

Fig. 3 SDS-polyacrylamide slab gel electrophoresis of hypodermin A in 5—15% gradient, pH 8.8. Standard proteins, 1 and 4 a, bovine serum albumin (68 000), b, aldolase (40 000), c, chymotrypsinogen (25 700), d, cytochrome *c* (11 700) After DE-32 cellulose column (2), after Sephadex G-100 (3)

Fig 4 Electroreduction of hypodermin A Sulfhydryl groups were determined by Ellman's reagent.

55°C, no loss of activity was observed during an interval of 10 min, but a drastic inactivation occurred above this temperature. At room temperature and 37°C the esterase was unchanged for 24 h.

The effect of pH on the activity is illustrated in Fig. 5B. The enzyme shows maximum activity in the pH range of 7—8.5.

Specificity. Hypodermin A exerts esterase and amidase activity on the same synthetic substrates as trypsin (BAEE, TAME and BAPA) with a 3-times higher specific activity with BAEE as substrate. It is without effect on chymotryptic substrate, containing an aromatic amino acid residue (BTEE). A special mention should be made on the titration of the proteinase by the active site titrant NPGB. At pH 8.3 the same burst of *p*-nitrophenyl as in the case of trypsin occurs from the hydrolysis of the substrate, whereas a 2-fold increase of the number of active sites per mg of hypodermin A is observed with the decrease of pH from 8.3 to 3.8.

The hydrolytic specificity on polypeptide substrates is examined with the B-chain of insulin and parvalbumin as substrates. In the case of the B-chain of insulin, the results are the same at two different times of incubation. the cleavage occurs only at one bond, at the carboxyl side of Arg_{22}. Under the conditions used, we cannot observe the cleavage of the Lys_{29}-Ala_{30} bond, typical for the action of trypsin.

A more complicated result is obtained when parvalbumin is used as substrate: this protein contains 14 lysine and one arginine residue(s). At least four bonds are cleaved, which excludes an analogy in specificity with clostripain [23].

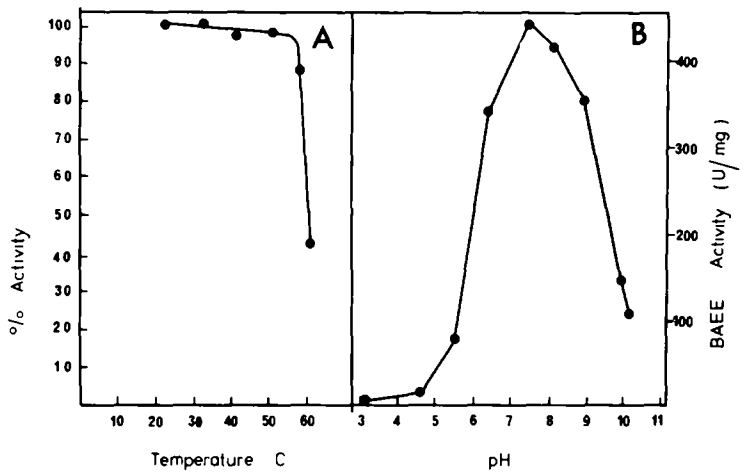

Fig. 5 Temperature stability at pH 8 5 after 10 min of incubation (A) and pH optimum of activity (B) of hypodermin A

Inhibitors. All trypsin inhibitors tested also inhibit hypodermin A; the results are summarized in Fig. 6A. The low molecular synthetic inhibitors of trypsin, diisopropyl fluorophosphate or TLCK (non-competitive) as well as benzamidine (competitive) act on the activity of the insect proteinase. The analogy with trypsin is strenghtened by the fact, that TPCK the aromatic inhibitor of chymotrypsin, is without action.

Results demonstrating the analogy with trypsin are also obtained with the natural polypeptide inhibitors as it can be seen in Fig. 6B. Hypodermin A is

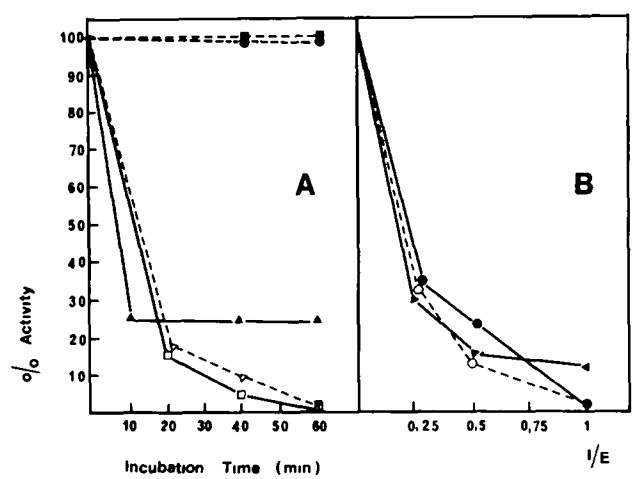

Fig 6. Inhibition of hypodermin A (enzyme concentration 10 nM in 0 05 M Tris-HCl/0.02 M $CaCl_2$ buffer, pH 7, at 25° C). (A) Effect of low molecular synthetic inhibitors. ■ - - - - - - ■, 1 mM EDTA, ● - - - - - - ●, 0.5 mM TPCK, ▲———▲, 0.25 mM benzamidine, □———□, 0.5 mM TLCK and △ - - - - - - △, 50 μM diisopropyl fluorophosphate (B) Effect of natural polypeptide inhibitors Percentage of remaining activities are expressed vs. inhibitor enzyme molar ratio ●———●, basic pancreatic trypsin inhibitor, ○ - - - - - - ○, soybean trypsin inhibitor and ▲———▲, ovomucoid

TABLE III

N-TERMINAL AMINO ACID SEQUENCE OF HYPODERMIN A AND COMPARISON WITH VARIOUS SERINE PROTEINASES

THR common amino acids to three and more proteins, Thr common amino acids to two proteins, *Thr* uncommon amino acids

	1				5				10				15						20	
Hypodermin A	ILE-	VAL-	GLY-	GLY-	*Val-*	GLU-	*Met-*	*Lys-*	*Ile-*	*Glu-*	*X-*	Phe-	PRO-	TRP-	*Glu-*	*Ile-*	*X-*	LEU-	Gln-	*X-*	*Pro*
Hypoderma collagenase [3]	ILE-	*Ile-*	Asn-	Gly-	Tyr-	GLU-	ALA-	*Tyr-*	*Thr-*	GLY-	Leu-	Phe-	PRO-	Tyr-	GLN-	*Ala-*	*Gly-*	LEU-	*Asp-*	*Ile-*	*Thr*
Trypsin [27]	ILE-	VAL-	GLY-	GLY-	Tyr-	*Thr-*	*Cys-*	*Gly-*	*Ala-*	*Asn-*	*Thr-*	*Val-*	PRO-	Tyr-	GLN-	VAL-	Ser-	LEU-	*Asn-*	*Ser-*	*Gly-*
Chymotrypsin [30]	ILE-	VAL-	Asn-	GLY-	*Glu-*	GLU-	ALA-	*Val-*	*Pro-*	GLY-	*Ser-*	*Trp-*	PRO-	TRP-	GLN-	VAL-	Ser-	LEU-	Gln-	*Asp-*	Lys
Thrombin [32]	ILE-	VAL-	GLY-	GLY-	*Gln-*	*Asp-*	ALA-	*Glu-*	*Val-*	GLY-	Leu-	*Ser-*	PRO-	TRP-	GLN-	VAL-	*Met-*	LEU-	*Phe-*	*Lys-*	Lys

completely inhibited both by the soybean trypsin inhibitor and the bovine pancreatic trypsin inhibitor, in a one to one molar ratio. Under the same conditions, the inhibition by ovomucoid is only 90%.

N-terminal sequence Results obtained by automatic Edman degradation are presented in Table III in which 18 amino acids, obtained without ambiguity, are compared with the amino acid terminal sequences of other serine proteinases and in particular with *H lineatum* collagenase.

Discussion

Hypodermin A together with a collagenase are the two major proteinases found in the digestive tract of the larvae of *H lineatum*. Its relative thermal stability helps us to understand why it accumulates intact during the 8 months of travelling of the larvae through the body of the host instead of being inactivated This stability is also favourable to the task of obtaining the homogeneous enzyme in few purification steps. At the early stage of the study, the inhibition studies indicated that hypodermin A belongs to the group of serine proteinases. It is inhibited by diisopropyl fluorophosphate but not by thiol reagents or EDTA This result, together with the susceptibility of hypodermin A to TLCK provides strong evidences that, in this enzyme, serine and histidine are both involved in the active center. The lack of inhibition by TPCK as well as the specific binding of the enzyme to natural peptidic trypsin inhibitor shows a possible similarity of its active center with that of trypsin rather than with that of chymotrypsin.

In terms of catalytic activity, hypodermin A is close but not identical to trypsin. Trypsin ester and amide substrates are well hydrolyzed in contrast to the corresponding chymotrypsin ester substrate. However, the specific activity of hypodermin A on BAEE is around 3-times higher than that of trypsin. During the titration of the active site, the same burst of acylation of the enzyme at neutral pH can be observed as in the case of trypsin with the same loss of deacylation rate However, at low pH (3 8) where hypodermin has no esterolytic activity, an increased number of active sites per mg are titrated which is quite different from the action of trypsin. A similar effect, e.g. the increase in titration value of active sites at acidic pH was also observed with cocoonase [7]. This could be related to the difference in the ionization of some residues in the binding pocket of the proteinases. More evidence for the difference from trypsin is that hypodermin A selectively cleaves the bond Arg_{22}-Gly_{23} in the B-chain of insulin without liberating the C-terminal alanine by the cleavage of the bond Lys_{29}-Ala_{30} as trypsin does.

Despite the phylogenic proximity, hypodermin A is no closer in amino acid composition to another insect proteinase, cocoonase from the silk moth (*Bombyx mori*) [7] than to serine proteinases from other sources. The most pronounced indication of the structural similarity of hypodermin A with the family of trypsin is the N-terminal sequence. In other serine proteinases these N-termini are created by the activation of their zymogens. It also seems to indicate that hypodermin A and *H lineatum* collagenase are formed from an inactive precursor, although at the present time, direct evidence is lacking. On the other hand, the structural homology of hypodermin A with a collagenase

from the same course indicates their development from a common ancestral gene product.

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References

1 Lienert, E and Throssel, W (1955) Expl Parasit 4, 117—122
2 Boulard, C. (1970) C Acad. Sci Paris 270, 1349—1351
3 Lecroisey, A , Boulard, C and Keil, B (1979) Eur J. Biochem 101, 385—393
4 Hurion, N., Fromentin, H and Keil, B (1977) Comp. Biochem Physiol 56B, 259—264
5 Nordwig, A. and Jahn, W F (1968) Eur J Biochem 3, 519—529
6 Jang, K D., Hang, H , Pfleiderer, G and Ishay, J (1978) Biochemistry 17, 4675—4682
7 Kafatos, F C , Law, J.H and Tartakoff, A M (1967) J. Biol Chem. 242, 1488—1494
8 Applebaum, S W , Birk, K , Harpoz, I and Bondi, A (1964) Comp Biochem Physiol 11, 85—91
9 Pfleiderer, G and Zwilling, R. (1966) Biochem Z 344, 127—140
10 Pechère, J.F , Demaille, J. and Capony, J P (1971) Biochim Biophys Acta 236, 391—408
11 Schwert, G.W and Takenaka, Y. (1955) Biochim Biophys. Acta 16, 570—575
12 Hummel, B C W. (1959) Can J. Biochem. Physiol. 37, 1393—1399
13 Erlanger, B F., Kokowsky, N and Cohen W (1961) Arch Biochem Biophys. 95, 271—278
14 Chase, T , Jr and Shaw, E. (1967) Biochem. Biophys Res Commun. 29, 508—514
15 Ames, G F.L (1974) J Biol Chem. 249, 634—644
16 Andrews, P (1964) Biochem. J 91, 222—233
17 Weitzman, P D.J (1976) Anal Biochem. 76, 170—176
18 Hirs, C H W (1967) Methods Enzymol. 11, 59
19 Liu, T Y and Chang, Y H (1971) J Biol Chem 246, 2842—2848
20 Hermodson, B A., Ericsson, L H , Titani, K , Neurath, H and Walsh, K.A (1972) Biochemistry 11, 4493—4502
21 Bridgen, J , Graffeo, A P., Karger, B L and Waterfield, M D. (1975) in Instrumentation in Amino Acid Sequence Analysis (Perham, R N , ed.), p 111, Academic Press, New York
22 Frank, G and Struber, W (1973) Chromatographia 6, 522—524
23 Gilles, A.-M , Imhoff, J -M and Keil, B (1979) J Biol. Chem. 254, 1462—1468
24 Lecroisey, A , De Wolf, A and Keil, B (1980) Biochem Biophys Res Commun 94, 1261—1265
25 Hruskas, J F , Law, J H and Kezdy, F (1969) J Biochem Biophys Res Commun 36, 272—277
26 Shotton, B M and Hartley, B S (1970) Nature (London) 225, 802—806
27 Walsh, K A and Neurath, H (1964) Proc Natl Acad Sci U.S.A 52, 884
28 Mikes, O , Holeysovsky, V , Tomasek, U and Sorm, F (1966) Biochem Biophys Res Commun 24, 346—352
29 Winter, W P. and Neurath, H (1970) Biochemistry 9, 4673—4679
30 Hartley, B.S (1964) Nature (London) 201, 1284—1287
31 Kafatos, F C , Tartakoff, A M and Law, J.H (1967) J Biol Chem 242, 1477—1487
32 Brown, J R and Hartley, B.S. (1966) Biochem. J 101, 214—228