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PROPERTIES AND SPECIFICITY OF THE MAJOR METAL-CHELATOR-SENSITIVE PROTEINASE IN THE KERATINOLYTIC LARVAE OF THE WEBBING CLOTHES MOTH

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Summary

The metal chelator-sensitive proteinase activity from the larvae of the webbing clothes moth, *Tineola bisselliella*, was fractionated into two components by chromatography on DEAE-cellulose and the properties of the major fraction investigated. The approximate molecular weight obtained by gel filtration was 24 000. The pH optimum of 9.4 and the high stability between pH 9.0 and 11.5 are consistent with the alkaline conditions known to be present in the larval mid-gut. The enzyme also showed a second region of high stability around pH 2.3. The cleavage specificity against *S*-carboxy-methyl A and B chains of insulin was quite different to that of the metal chelator-sensitive proteinases from snake venoms and microorganisms. 10 bonds in the A-chain and 8 bonds in the B-chain were cleaved and the tentative rules governing the specificity limitations of this metal-chelator-sensitive proteinase are discussed.

Introduction

In a previous paper [1] the identification and partial resolution of the complex mixture of proteinases and peptidases present in the larvae of the webbing clothes moth, *Tineola bisselliella*, were described. About 50% of the proteinase activity was unaffected by diisopropylfluorophosphate (Dip-F) or thiol reagents but was inhibited by the metal chelators EDTA and 1 : 10 phenanthroline. This activity was tentatively termed a metallo-proteinase. Chromatography of larval extracts on ion-exchange and Sephadex columns and to a lesser extent electrophoresis in acrylamide gels, indicated only a single peak of metal-chelator-sensitive proteinase activity [1].

* Abbreviations used are: Ac-Tyr, *N*-acetyl-DL-tyrosine; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Bz-Arg-NapN, *N*-benzoyl-DL-arginine β-naphthylamide; Bz-Arg-NAn, *N*-benzoyl-DL-arginine *p*-nitroanilide; Dip-F, diisopropylfluorophosphate; Diol buffer, 2-amino-2-methyl-1,3-propandiol buffer; S-Cm, *S*-carboxymethyl.

In this report the further fractionation of this material into two metal-chelator-sensitive proteinases is described, along with observations on the peptide-bond specificity and other properties of the major metal-chelator-sensitive component.

Materials and Methods

Chemicals

Chemicals used and their sources were: *N*-acetyl-DL-tyrosine (Ac-Tyr), *N*-acetyl-DL-tyrosine ethyl ester (Ac-Tyr-OEt) and *N*-benzoyl-DL-arginine *p*-nitroanilide (Bz-Arg-NAn), Schwarz-Mann Research Laboratories; *N*-benzoyl-DL-arginine β-naphthylamide (Bz-Arg-NapN), Calbiochem; Dip-F, Aldrich Chemical Co., bovine serum albumin, Sigma Chemical Co.; fast garnet GBC, G.T. Gurr Ltd., England; Hammarsten casein, E. Merck, Germany; DEAE-cellulose (DE 52), Reeve Angel and Co., England. The S-carboxymethyl A- and B-chains of bovine insulin were gifts from Dr I.J. O'Donnell of this laboratory.

Enzyme

The starting material for this study was fraction B4, obtained as described in the previous paper [1]. This fraction contained most of the metal-chelator-sensitive proteinase of *T. bisselliella* extracts and considerable trypsin-like activity but only small amounts of chymotrypsin-like activity and carboxypeptidase activity and no aminopeptidase activity. This fraction had been stored in 50% glycerol at −20° C, the glycerol being removed by ultrafiltration before further chromatography.

Enzyme assays, protein determination, column chromatography and ultrafiltration were as described in the previous paper [1].

Acrylamide gel electrophoresis

Disc gel electrophoresis was carried out as described previously [1]. In addition micro-gels were run in conventional apparatus in 1.5 mm i.d. × 100 mm capillary tubes (Drummond 200 µl Microcaps) containing 160 µl running gel and 10 µl stacking gel. These were connected by PVC tubing to a 0.5 ml Eppendorf pipette tip as described by Burr et al. [2]. Initially the gels were removed from the capillary tubing by cracking on immersion in an acetone-dry ice mixture [2]. However the degree of glass fragmentation was quite variable and gels were more easily removed by extrusion with a close fitting wire rod (Drummond 100 µl Wiretrol plunger). To minimize wall effects the capillary tubes were pretreated with 1% dimethyl-dichlorosilane.

The fixation and staining of protein bands and the detection of trypsin-like enzymes in acrylamide gels was as described previously [1]. Metal-chelator-sensitive proteinase activity was detected directly in acrylamide gels by a modification of the method of Uriel [3]. The gel was soaked for 90 min at 37° C in a substrate solution of urea denatured cytochrome *C* (2% w/v) [4] and 33 mM 2-amino-2-methyl-1,3-propandiol (Diol) buffer (pH 9.4). The gel was then withdrawn from the substrate solution and incubated in a capped tube for a further 2 h at 37° C. The proteinase activity bands were visualized by placing the gel in 12.5% trichloroacetic acid and appear as clear zones against an evenly

precipitated background. The gels were photographed under reflected light against a black background. Electrophoretic mobilities are expressed relative to bromphenol blue.

Peptide bond specificities of metal-chelator-sensitive proteinase on A and B chains of insulin

Digestion. Since fraction B4f showed residual trypsin-like activity it was pretreated with Dip-F before adding to the digestion mixture. The pre-treatment reaction mixture (0.20 ml) contained: Diol buffer, pH 9.5, 1.0 μmol; Dip-F, 3.4 μmol and B4f proteinase 110 μg. This was held at 5° C for 60 min and then added to a solution (2.0 ml) containing: Diol buffer, pH 9.5, 10 μmol and either S-Cm-A-chain insulin, 8.0 mg or S-Cm-B-chain insulin, 10.0 mg, and incubated for 2 h at 37° C. Each digestion and analysis of products was carried out in duplicate.

Analysis of peptides. The lyophilized digestion products were dissolved in 0.4 ml 0.5% ammonia, applied as 10—30 cm strips across sheets of Whatman 3 MM paper and subjected to high voltage electrophoresis [1] at pH 3.5 for A-chain digests and pH 6.5 for B-chain digests. The bands were located by staining 5-mm side strips with the ninhydrin/collidine reagent [5]. Peptides were eluted with 0.5% ammonia, dried and checked for purity by high voltage electrophoresis at pH 3.5 and 6.5 and chromatography in BAWP (butanol/acetic acid/water/pyridine, 15 : 3 : 12 : 10) [6].

Where necessary further purification was effected by chromatography in BAWP or electrophoresis. Electrophoretic mobilities (m) are expressed as fractions of the distance between ε-DNP-lysine and aspartic acid and refer to the position of the leading edge of the migrating spot [7].

Amino acid analysis. Peptides were hydrolysed in the presence of 1.0 μmol thioglycollic acid, with 0.5 ml of twice distilled 6 M HCl for 24 h at 110° C in sealed and evacuated tubes. Amino acid analyses were carried out on a Beckman 120C amino acid analyzer.

Results

Further purification of metal-chelator-sensitive proteinase

As shown in Fig. 1, fraction B4 was separated into two peaks of metal-chelator-sensitive proteinase activity (f and g) which were free of chymotrypsin-like and carboxypeptidase activity but contaminated with residual trypsin-like activity. Examination by acrylamide gel electrophoresis showed that fraction B4f consisted of a major protein band, R_m 0.55 and three minor bands, while the minor metal-chelator-sensitive proteinase, fraction B4g, consisted of a single protein band, R_m 0.52 (Fig. 2). The non-identity of the major protein bands of these two fractions was verified by acrylamide gel electrophoresis in micro gel rods (1.5 mm × 80 mm) and by running adjacent samples in acrylamide gel slabs (70 mm × 70 mm × 3 mm). The specific activities of fractions B4f and B4g were 212.7 $[PU]^{278}$/mg and 265 $[PU]^{278}$/mg, respectively which represent an approximate 3-fold purification over fraction B4 and a 115—143-fold purification over the original 20 000 × g supernatant [1].

Although fraction B4f is not homogeneous acrylamide gels show that the

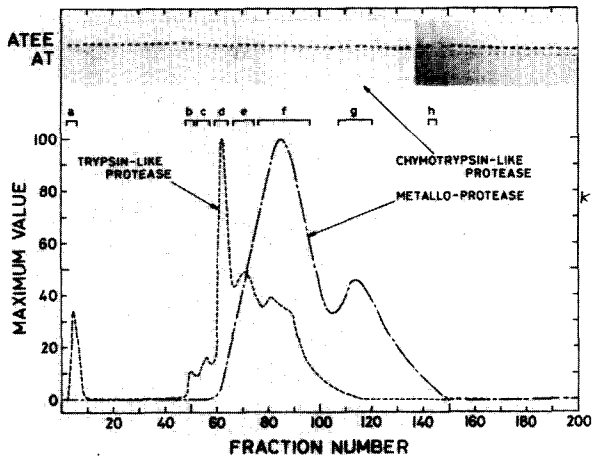

Fig. 1. Chromatography of fraction B4 on DEAE-cellulose column (0.9 cm × 15 cm). Flow rate was 8.7 ml/h, fraction volumes were 2.9 ml. Sample (6.0 ml), was pumped on followed by 45 ml of starting buffer (0.01 M Tris/Cl, pH 7.3). At tube 18 a linear gradient was established from 0.01 M Tris/Cl pH 7.3 (200 ml) to 0.2 M NaCl in the same buffer (200 ml). At tube 144, a second linear gradient was established from 0.2 M NaCl (100 ml) to 0.3 M NaCl (100 ml) both in 0.01 M Tris/Cl pH 7.3. Every second tube was assayed for metallo-protease activity, trypsin-like activity, chymotrypsin-like activity and carboxypeptidase activity and the results expressed as a percentage of the maximum activity per tube obtained during the fractionation. These maximum values were: metallo-protease, 30.7 $[PU]^{278}$; trypsin-like activity, 1.04 μmol/90 min. Chymotrypsin-like activity was not quantitated but was monitored by the high voltage electrophoresis spot test. No carboxypeptidase activity was recovered from the column. Tube contents were pooled to give fractions B4a, B4b, B4c, B4d, B4e, B4f, B4g and B4h as shown and concentrated by ultrafiltration.

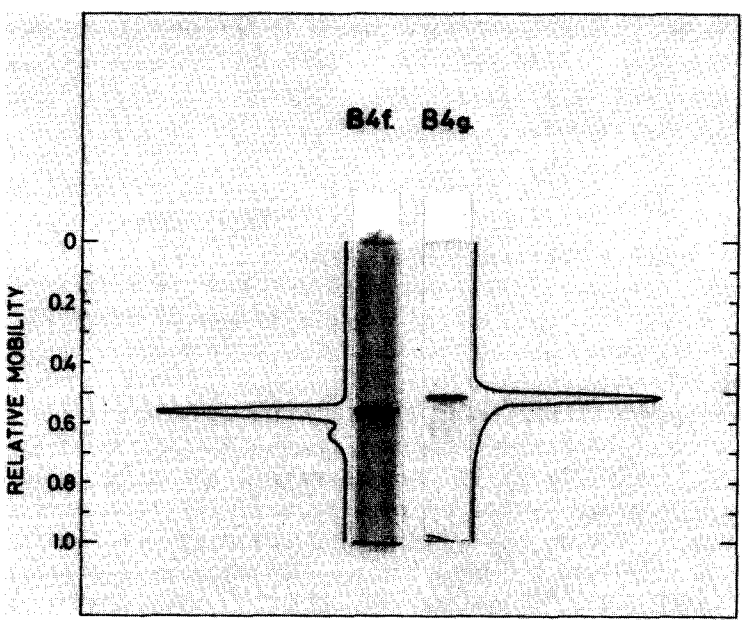

Fig. 2. Protein bands in fractions B4f and B4g following electrophoresis in discontinuous acrylamide gels. The running gel contained 7.5% acrylamide. Migration is towards the anode, and is expressed relative to the mobility of bromphenol blue. Densitometer traces of the scanned gels are also shown.

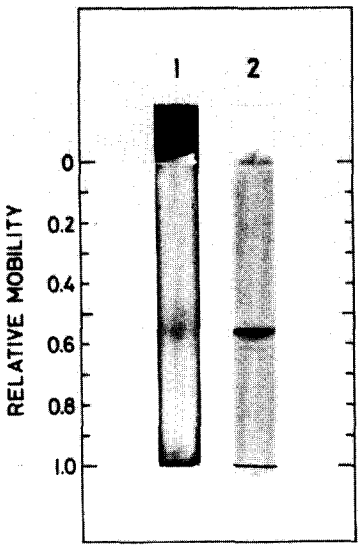

Fig. 3. Direct detection of fraction B4f metallo-proteinase activity in acrylamide gels after impregnation with denatured cytochrome *C*. Electrophoresis was in 7.5% gels and detection of proteinase activity was as described in the methods. A reference gel stained for protein is also shown, gel 2. Migration is towards the anode.

Dip-F-insensitive, metal-chelator-sensitive proteinase activity coincides with the major protein band at R_m 0.55 (Fig. 3).

Properties of the major metal-chelator-sensitive proteinase (fraction B4f)

Molecular weight. The metal-chelator-sensitive proteinase in B4f eluted from a Sephadex G-200 column, calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c* [8], at a position corresponding to a molecular weight of 24 000.

Casein digestion. The standard curve of enzyme concentration against the degree of casein digestion, as measured by the absorbance at 278 nm, $[PU]^{278}$, or colorimetrically with the Folin phenol reagent, $[PU]^{F}$ is shown in Fig. 4. One $[PU]^{278}$ is equivalent to 0.343 $[PU]^{F}$ and 4.7 μg of fraction B4f. The two protease units $[PU]^{278}$ and $[PU]^{F}$ are not equivalent because of the unequal relative contributions of tyrosine and tryptophan to ultraviolet absorption and colour formation [9].

pH optimum. The effect of pH on the hydrolysis of casein by the metal chelator-sensitive proteinase in fraction B4f is shown in Fig. 5. The pH optimum was pH 9.4, which is very close to that (9.3) reported by Duspiva [10] for the total proteinase activity of extracts of mid-gut epithelia.

Stability. The effect of pH on the stability of the metal chelator-sensitive proteinase in fraction B4f is shown in Fig. 6. Two regions of maximum stability were obtained. One was at pH 9.0—11.0 with maximum stability at pH 10.3. The other was over a much narrower range at acid pH with maximum stability at pH 2.3. Kafatos et al. [11] similarly reported two pH regions of maximum stability for the serine-proteinase coocoonase but found that at the acid pH maxima, coocoonase was remarkably stable to high temperatures. As shown in

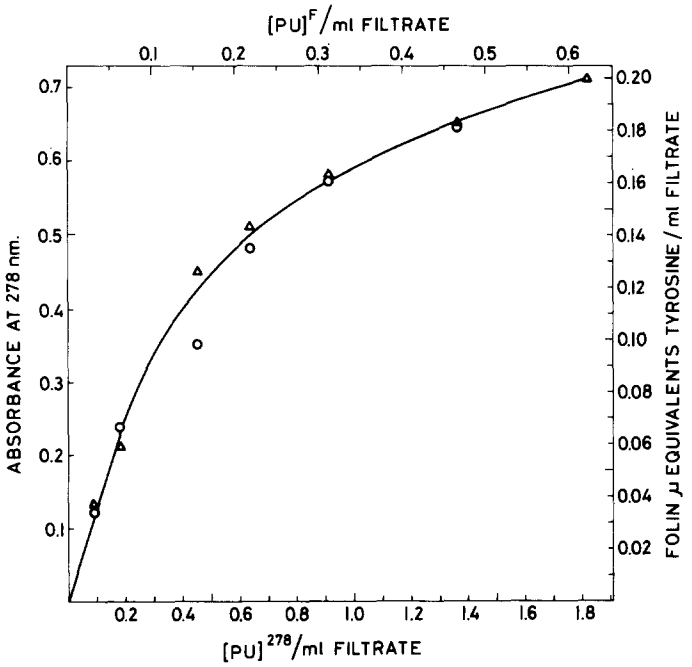

Fig. 4. Standard curve of enzyme concentration against digestion products [40] as measured by the extinction of 278 nm, [PU] ^{278}, △, or with the Folin phenol reagent, [PU] ^{F}, ○. The initial slope of the standard curve is equal to the production of 1 μequiv of tyrosine/30 min/proteinase unit at pH 9.5 and 37°C. One [PU] ^{278} is equivalent to 0.343 [PU] ^{F} and 4.7 μg of fraction B4f.

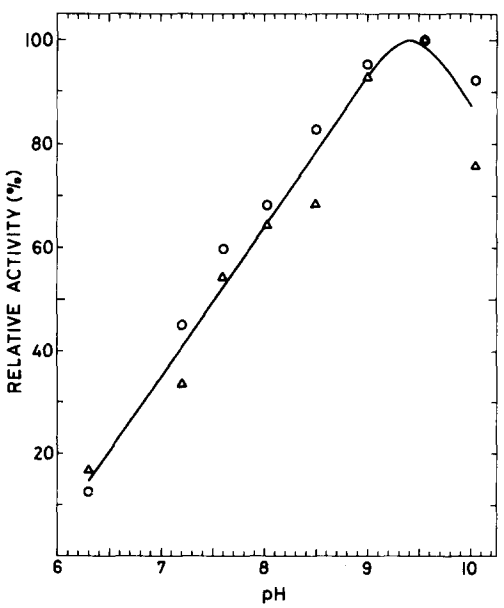

Fig. 5. Effect of pH on the metal-chelator-sensitive proteinase (B4f) activity expressed as [PU] ^{278}, △, or [PU] ^{F}, ○. Buffers used were 0.05 M Na_2HPO_4 pH 6.3 and 7.2; 0.05 M Diol buffer pH 7.6—10.05.

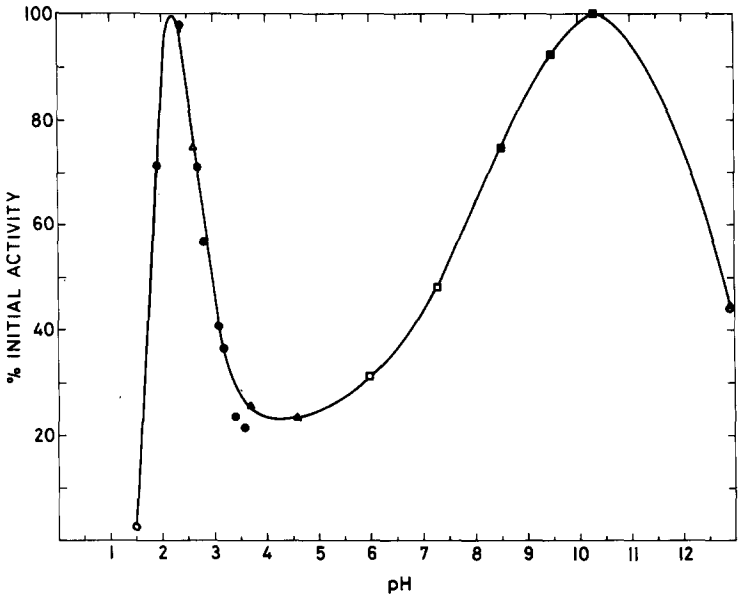

Fig. 6. pH stability of the metal-chelator-sensitive proteinase B4f. Enzyme was held at the pH indicated for 24 h at 4°C, then casein was added and the enzyme assayed for residual activity at pH 9.5 and 37°C. The buffers used at 0.05 M were: HCl, ○; glycine/HCl, ●; HCl/KCl, △; sodium acetate, ▲; sodium phosphate, □; Diol buffer, ■; KOH; ⊕.

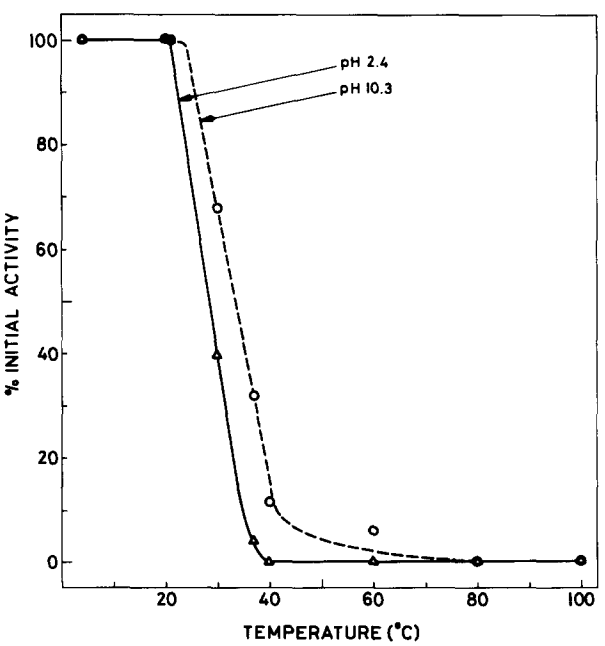

Fig. 7. Temperature stability of metal-chelator-sensitive proteinase at pH 2.4 and 10.3. Enzyme was pre-incubated at appropriate temperature for 30 min, then cooled to 37°C and assayed for residual proteinase activity at pH 9.5 [1].

Fig. 7, the *Tineola* enzyme, did not show increased stability to elevated temperatures at pH 2.4, and was actually more sensitive to increasing temperature at pH 2.4 than at pH 10.3.

Inhibition of fraction B4f metal-chelator-sensitive proteinase by EDTA and metal ions

As shown in Table I, EDTA completely inhibits the proteinase in fraction B4f and this inhibition is due to the chelating properties of EDTA. When a complex of the metal chelator and calcium is formed prior to exposure of the enzyme, negligible inhibition of protease activity occurs. The inhibition of EDTA can be partially reversed by the addition of Ca^{2+}, Zn^{2+} and to a lesser extent Mg^{2+} but not by Cu^{2+} or Cr^{3+}. These metal ions themselves either did not inhibit (Ca^{2+}, Mg^{2+}) or only partially inhibited (Cu^{2+}, Zn^{2+}, Cr^{2+}) proteinase activity (Table II).

Specificity of metal-chelator-sensitive proteinase in the cleavage of insulin chains

The peptides obtained when S-Cm-A-chain insulin was digested with fraction B4f metal chelator-sensitive proteinase are shown in Fig. 8. The details on the amino acid compositions, yields, electrophoretic mobilities and isolation procedures for these peptides are given in a supplement*.

The yields for isoleucine and to a lesser extent valine in peptides 3, 8c, 9a, 11 and 13 following 24 h hydrolysis were low as reported by Harfenist [12]. From its amino acid composition, peptide 2 could be either Leu_{13}-Tyr_{14}-Gln_{15} or Tyr_{14}-Gln_{15}-Leu_{16}. N-terminal analysis of peptide 2 by the Dansyl method [13] showed leucine was the N-terminal residue and that peptide 2 is Leu_{13}-Tyr_{14}-Gln_{15}. Thus peptide 4 is assumed to be Leu_{16}, not Leu_{13}. The

TABLE I

EDTA INHIBITION OF B4f PROTEINASE AND ITS REVERSAL BY METAL IONS

The enzyme was incubated at pH 9.4 in 0.3 ml reaction mixture containing 0.5 μmol EDTA or calcium—EDTA complex for 15 min at 30°C (First treatment). 1.0 μmol metal ion was then added and the incubation continued for a further 15.0 min (Second treatment). The residual proteinase activity was determined at 37°C and pH 9.4 following the addition of 0.1 ml of 2% (w/v) casein, as described previously [1].

First treatment	Second treatment	Realtive activity (%)
—	—	100
Ca·EDTA complex	—	90
EDTA	—	0
EDTA	$CaCl_2$	55
EDTA	$ZnCl_2$	50
EDTA	$MgCl_2$	20
EDTA	$CuCl_2$	1
EDTA	$CrCl_3$	0

* Supplementary data to this article, giving details of the insulin S-Cm-B chain peptides are deposited with, and can be obtained from: Elsevier Scientific Publishing Co., BBA Data Deposition, P.O. Box 1527, Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/021/67431/384 (1975) 215.

TABLE II

EFFECT OF METAL IONS ON B4f METALLO-PROTEINASE ACTIVITY

The enzyme was pre-incubated with 0.0017 M metal ion for 30 min at pH 9.4 and 30°C, then casein was added and the residual activity determined at 37°C as described previously [1].

Additions	Relative activity (%)
None	100
$CaCl_2$	103
$MgCl_2$	114
$CuCl_2$	86.7
$ZnCl_2$	76.5
$CrCl_3$	74.4
$NiCl_2$	49.1
$CoCl_3$	45.6
$MnSO_4$	37.9
$HgCl_2$	0

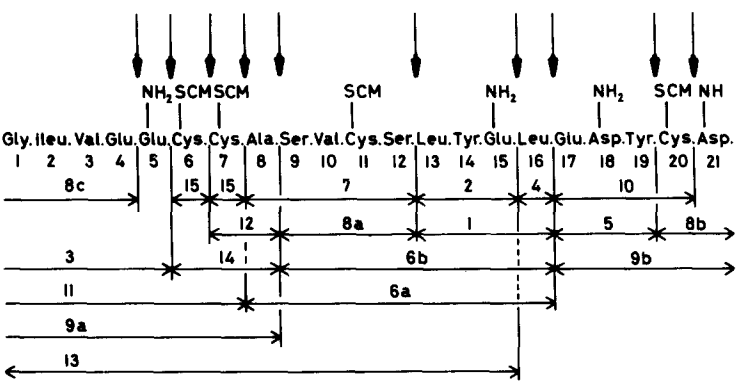

Fig. 8. Sites of cleavage of S-Cm-A-chain insulin by the metal-chelator-sensitive proteinase (fraction B4f) from *T. bisselliella*. Deductions were made from the data in the supplement.

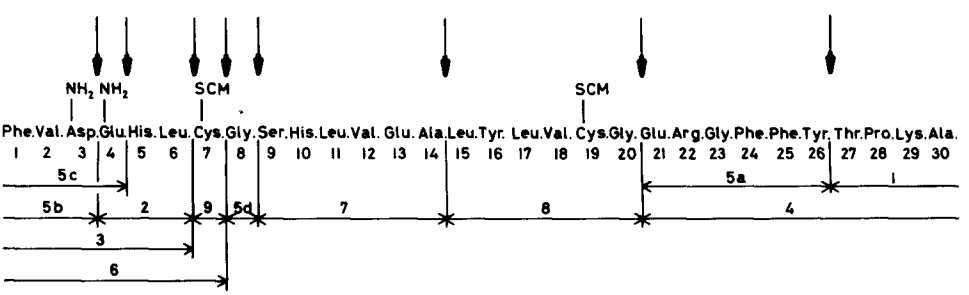

Fig. 9. Sites of cleavage of SCM-B-chain insulin by the metal chelator-sensitive proteinase (fraction B4f) from *T. bisselliella*. Deductions were made from the data in the supplement.

minor peptide 13 confirms that cleavage has occurred at the Gln_{15}-Leu_{16} bond.

The peptides obtained when S-Cm-B-chain insulin was digested with the metal-chelator-sensitive proteinase (fraction B4f) are shown in Fig. 9. The data characterising these peptides are given in the supplement.

Peptide 9 (SCMcys) could have come from position 7 or 19 and peptide 5c (glycine) could have come from position 8, 20 or possibly 23. Since no peptides were obtained to suggest cleavage at the Val_{18}-Cys_{19}, Cys_{19}-Gly_{20}, Arg_{22}-Gly_{23}, or Gly_{23}-Phe_{24} bonds, these peptides 9 and 5c are assumed to represent the residues Cys_7 and Gly_8, respectively although it is possible they also represent Gly_{20} and to a lesser extent Cys_{19}.

Discussion

The present results demonstrate that the metal-chelator-sensitive proteinase activity of *T. bisselliella* can be fractionated into two components by ion-exchange chromatography and the non-identity of the two fractions has been confirmed by acrylamide gel electrophoresis in micro-gel rods, gel rods and gel slabs. Several of the properties of the major component (B4f) have been investigated.

Its inhibition by EDTA is dependent on the chelating properties of the inhibitor since the calcium · EDTA complex had no effect on activity. This inhibition by EDTA can be partially reversed by the addition of a slight excess of free calcium or zinc ions.

The molecular weight of approximately 24 000 is much less than of 35 000—40 000 reported for the metal chelator-sensitive proteinases of microbial origin, but similar to that (20 000—26 000) found for the serine proteinases of microbial and higher animal origins (see ref. 14 for review) and a metal-chelator-sensitive proteinase from snake venom [15].

The pH optima of 9.4 and the high stability of this enzyme between pH 9.0 and 11.5 are consistent with the observations on the alkalinity of the larval mid-gut, where most digestion occurs [16—18].

The specificity of the metal-chelator-sensitive proteinase from *Tineola* is quite different to that of the metal-chelator-sensitive proteinases from microorganisms [19—22] and several snake venoms [15,23—25]. These enzymes primarily attacked the bonds His_5-Leu_6, His_{10}-Leu_{11}, Ala_{14}-Leu_{15}, Tyr_{16}-Leu_{17}, Leu_{17}-Val_{18}, Gly_{23}-Phe_{24}, Phe_{24}-Phe_{25} and Phe_{25}-Tyr_{26}, of insulin B-chain with additional cleavages for some of these enzymes. Of all these bonds, only the Ala_{14}-Leu_{15} bond was cleaved by the *Tineola* metal-chelator-sensitive proteinase. With regard to the other B-chain bonds cleaved by the *Tineola* enzyme, Asn_3-Gln_4, Cys_7-Gly_8, Gly_{20}-Glu_{21} and Tyr_{26}-Thr_{27} are each cleaved by several but not all of the metal-chelator-sensitive proteinases from *Serratia marcescens* [26], *Proteus mirabilis* [27] and *Aspergillus ochraceus* [28]. The Gln_4-His_5 bond is not cleaved by the metallo-proteinases from any of the microorganisms and the Leu_6-Cys_7 bond is only cleaved by that from *A. ochraceus*. Six of the eight bonds of SCM-B-chain cleaved by the *Tineola* metal-chelator-sensitive proteinase were also cleaved by a Dip-F-insensitive, thiol reagent-insensitive protease from the crayfish *Orconectes virilis*

[29]. This latter enzyme however had a very broad specificity and cleaved 21 of the 29 bonds in insulin B-chain.

The rules governing the specificity of the *Tineola* metal-chelator-sensitive proteinase are not obvious. The residues donating the carboxyl or amino groups to the susceptible bonds have no common features and range from small residues such as glycine, alanine and serine, to large bulky residues such as leucine, tyrosine, glutamine and asparagine, or large charged residues such as glutamic acid or carboxymethyl cysteine. However, it is well established for some proteinases that residues other than those involved in the cleaved peptide bonds, are important determinants of proteinase specificity. Thus subtilisin cleavage has been shown to be influenced by the three amino acid residues on both sides of the hydrolysed bond [30] and papain [31] and other thiol proteinases [32] are thought to have seven substrate binding sub-sites. For thiol proteinases, the dominant feature of their specificity was the requirement for a bulky side chain on the amino acid adjacent (on the amino terminal side) to the residue contributing the carbonyl group to the susceptible bond [31—33].

Consideration of the eight successive residues $P_4 \cdot P_3 \cdot P_2 \cdot P_1 - P'_1 \cdot P'_2 \cdot P'_3 \cdot P'_4$ around the $P_1 - P'_1$ bonds in A and B chains of insulin that were hydrolysed by the *Tineola* metal-chelator-sensitive proteinase (Table III) indicates that residues P_3 and P_2 may have greatest influence on the cleavage specificity. In all bonds hydrolysed both positions P_3 and P_2 are occupied by residues with bulky side chains. In 14 out of the 18 cases, P_3 was occupied by the bulky hydrophobic residues leucine, isoleucine, valine or phenylalanine (10 times) or histidine, glutamine and asparagine (4 times) the histidine being uncharged at the pH (9.4) of the *Tineola* enzyme. In 11 out of the 18 cases P_2

TABLE III

RESIDUES ADJACENT TO BONDS CLEAVED BY METALLOPROTEASE

Insulin chain	Residues	P_4 — P_3 — P_2 — P_1 ↓ P'_1 — P'_2 — P'_3 — P'_4
A	16—21	Leu — Glu — Asn — Tyr — Cys — Asn
A	3—10	Val — Glu — Gln — Cys — Cys — Ala — Ser — Val
A	5—12	Gln — Cys — Cys — Ala — Ser — Val — Cys — Ser
A	2— 9	Ile — Val — Glu — Gln — Cys — Cys — Ala — Ser
B	11—18	Leu — Val — Glu — Ala — Leu — Tyr — Leu — Val
B	17—24	Leu — Val — Cys — Gly — Glu — Arg — Gly — Phe
A	9—16	Ser — Val — Cys — Ser — Leu — Tyr — Gln — Leu
B	1— 8	Phe — Val — Asn — Gln — His — Leu — Cys — Gly
A	13—20	Leu — Tyr — Gln — Leu — Glu — Asn — Tyr — Cys
B	5—12	His — Leu — Cys — Gly — Ser — His — Leu — Val
A	12—19	Ser — Leu — Tyr — Gln — Leu — Glu — Asn — Tyr
A	1— 8	Gly — Ile — Val — Glu — Gln — Cys — Cys — Ala
B	23—30	Gly — Phe — Phe — Tyr — Thr — Pro — Lys — Ala
B	1— 7	Phe — Val — Asn — Gln — His — Leu — Cys
A	4—11	Glu — Gln — Cys — Cys — Ala — Ser — Val — Cys
B	3—10	Asn — Gln — His — Leu — Cys — Gly — Ser — His
A	17—21	Glu — Asn — Tyr — Cys — Asn
B	4—11	Gln — His — Leu — Cys — Gly — Ser — His — Leu

was occupied by a residue with a side chain carbonyl group (carboxymethylcysteine, 5 times; glutamic acid, glutamine and asparagine, each 2 times). The ability to form hydrogen bonds may be an important requirement for the residue in position P_2. Hydrogen bonding has been implicated in the orientation of susceptible peptide bonds in chymotrypsin and subtilisin catalyzed cleavages [34].

Although there appear to be no common characteristics among the residues found in positions P_1 and P_1' of hydrolysed bonds, some residues, particularly valine and phenylalanine, are absent from these positions. None of the peptide bonds involving valine (10 bonds), phenylalanine (4 bonds) and isoleucine, proline, lysine and arginine (2 bonds each) were cleaved. Valine cannot be tolerated in position P_1 of bonds hydrolysed by papain [31] and presumably ficin, bromelain [35,36] and the Chinese gooseberry proteinase [32] and the inability of most proteolytic enzymes to hydrolyse bonds involving proline is well established.

In summary, peptide bond hydrolysis by the *Tineola* metal-chelator-sensitive proteinase is favoured if position P_3 is occupied by an amino acid residue with a bulky, preferably hydrophobic side chain, position P_2 is occupied by a residue with a bulky side chain capable of participating in hydrogen bonds and if positions P_1 and P_1' are not occupied by valine or phenylalanine, and possibly isoleucine, proline, lysine or arginine. The validity of these tentative rules and the possible requirements or limitations for residues in the other positions P_4, and P_2' - P_4' will be verified by using model peptide substrates and other proteins of known sequence. The minor metal-chelator-sensitive proteinase in fraction B4g of *T. bisselliella* has almost identical cleavage specificity to that reported here for the B4f enzyme (Ward, C.W., unpublished).

From the limited number of investigations to date, it appears that invertebrates in general lack a pepsin-like enzyme, and the specificities of their proteinases would be expected to compensate for such a lack [37,38]. The metal-chelator-sensitive proteinases of *T. bisselliella*, though having a specificity quite different to that of pepsin-like enzymes [39], very effectively complement the expected limited specificities of the trypsin-like and chymotrypsin-like enzymes present in these larvae.

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References

1 Ward, C.W. (1975) Aust. J. Biol. Sci. 28, 1—23
2 Burr, I.M., Balanr, L., Stauffacher, W. and Renold, A.E. (1970) Anal. Biochem. 35, 82—86
3 Uriel, J. (1960) Nature 188, 853—854
4 Laskowski, M. (1955) Method. Enzymol. 2, 26—36
5 Bennet, J.C. (1967) Method. Enzymol. 11, 330—339
6 Waley, S.G. and Watson, J. (1953) Biochem. J. 55, 328—337
7 Offord, R.E. (1966) Nature 211, 591—593

8 Andrews, P. (1965) Biochem. J. 96, 595—606
9 Northrop, J.H., Kunitz, M. and Herriott, R.M. (1948) Crystalline Enzymes, 2nd ed., p. 306, Columbia University Press, New York
10 Duspiva, F. (1936) C.R. Trav. Lab. Carlsberg Ser. Chim. 21, 177—202
11 Kafatos, F.C., Tartakoff, A.M. and Law, J.H. (1967) J. Biol. Chem. 242, 1477—1487
12 Harfenist, E.J. (1953) J. Am. Chem. Soc. 75, 5528—5533
13 Gray, W.R. (1967) Method. Enzymol. 11, 139-151
14 Matsubara, H. and Feder, J. (1971) in The Enzymes (Boyer, P.D., ed.), 3rd ed., Vol. 3, pp. 721—795, Academic Press, New York
15 Spiekerman, A.M., Fredericks, K.K., Wagner, F.W. and Prescott, J.M. (1973) Biochim. Biophys. Acta 293, 464—475
16 Day, M.F. (1951) Aust. J. Sci. Res. Ser. B 4, 42—48
17 Waterhouse, D.F. (1952a) Aust. J. Sci. Res. Ser. B 5, 143—168
18 Waterhouse, D.F. (1952b) Aust. J. Sci. Res. Ser. B 5, 178—188
19 Morihara, K., Tsuzuki, H. and Oka, T. (1968) Arch. Biochem. Biophys, 123, 572—588
20 Millet, J. and Archer, R. (1966) Eur. J. Biochem. 9, 456—462
21 Matsubara, H., Sasaki, R., Singer, A. and Jukes, T.H. (1966) Arch. Biochem. Biophys. 115, 324—331
22 Morihara, K. and Tsuzuki, H. (1966) Biochim. Biophys. Acta 118, 215—218
23 Satake, M., Omori, T., Iwanaga, S. and Suzuki, T. (1963) J. Biochem. (Tokyo) 54, 8—16
24 Pfleiderer, G. and Kraus, A. (1965) Biochem. Z. 342, 85—94
25 Takahashi, T. and Ohsaka, A. (1970) Biochim. Biophys. Acta 198, 293—307
26 Braymer, H.D., Davidson, P.K. and Larson, A.D. (1968) Abstr. 156th Meeting Am. Chem. Soc. Biol. No. 51
27 Hampson, S.E., Mills, G.L. and Spencer, T. (1963) Biochim. Biophys. Acta 73, 476—487
28 Kishida, T. and Yoshimura, S. (1964) J. Biochem. Tokyo 55, 95—101
29 De Villez, E.J. and Lau, J. (1970) Int. J. Biochem. 1, 108—114
30 Morihara, K., Tsuzuki, h. and Oka, T. (1971) Biochem. Biophys. Res. Commun. 42, 1000—1006
31 Berger, A. and Schechter, I. (1970) Phil. Trans. Roy. Soc. London Ser. B 257, 249—264
32 McDowall, M.A. (1973) Biochim. Biophys. Acta 293, 226—231
33 Gerwin, B.I., Stein, W.H. and Moore, S. (1966) J. Biol. Chem. 241, 3331—3339
34 Wright, C.S. (1972) J. Mol. Biol. 67, 151—163
35 Husain, S.S. and Lowe, G. (1970) Biochim. J. 117, 341—346
36 Lowe, G. and Yuthavong, Y. (1971) Biochem. J. 124, 107—115
37 De Villez, E.J. (1968) Am. Zool. 8, 541
38 Huggins, A.K. and Munday, K.A. (1968) in Advances in Comparative Physiology and Biochemistry (Lowenstein, O., ed.), Vol. 3, p. 323, Academic Press, New York
39 Fruton, J.S. (1971) in The Enzymes (Boyer, P.D., ed.), 3rd ed., Vol. 3, pp. 119—164, Academic Press, New York
40 Matsubara, H. (1970) Method. Enzymol. 19, 642—651