Aust. J. Biol. Sci., 1975, **28**, 439–45

Properties and Specificity of a Second Metal Chelator-sensitive Proteinase in the Keratinolytic Larvae of the Webbing Clothes Moth

Colin W. Ward

Division of Protein Chemistry, CSIRO, Parkville, Vic. 3052.

Abstract

The properties of a second metal chelator-sensitive proteinase (metalloproteinase 2) from the larvae of the webbing clothes moth, *Tineola bisselliella*, have been studied. The pH optimum for casein digestion was 9·4 and the enzyme showed high stability between pH 8 and 11, but very poor stability at acid pH. The proteinase was inhibited by EDTA, but not by an EDTA–calcium complex. EDTA inhibition could be reversed by addition of a slight excess of calcium or zinc ions. The cleavage specificity of metalloproteinase 2 against the A and B chains of *S*-carboxymethyl insulin was almost identical to that found previously for metalloproteinase 1.

Introduction

In an earlier paper (Ward 1975*a*), *Tineola bisselliella* larvae were shown to contain considerable metal chelator-sensitive proteinase activity in addition to other proteinases and peptidases. This activity was subsequently fractionated into two metal chelator-sensitive components and some of the properties of one of these components, including its peptide bond specificity on both chains of insulin, have been described (Ward 1975*b*).

In this paper the properties and peptide bond specificity of the second component are described. Evidence is also presented to show that these are the only major metal chelator-sensitive proteinases present in extracts of these larvae.

Materials and Methods

The methods employed for enzyme assays, protein determination, column chromatography, ultrafiltration and acrylamide gel electrophoresis were as described by Ward (1975*a*). The methods used for electrophoresis in micro-acrylamide gels, direct detection of proteinase activity in acrylamide gels and the determination of peptide bond cleavage specificities with S-Cm*-A and -B chains of insulin were as described by Ward (1975*b*).

The starting materials used for this study were fractions C4 and B4g obtained as described by Ward (1975*a*) and Ward (1975*b*) respectively.

Results

Further Purification of Metal Chelator-sensitive Proteinase

When fraction C4 was chromatographed on a column of DEAE-cellulose at pH 7·3 (Fig. 1), the metal chelator-sensitive proteinase activity was effectively separated from the carboxypeptidase and chymotrypsin-like activities but still

* Abbreviations used are: DFP, diisopropylfluorophosphate; S-Cm, *S*-carboxymethyl; Diol buffer, 2-amino-2-methyl-1,3-propandiol buffer.

contaminated with a low level of trypsin-like proteinase activity. Only a single peak of metal chelator-sensitive proteinase was obtained during this fractionation, in contrast to the two peaks of activity obtained when fraction B4 was similarly chromatographed (Ward 1975*b*). The elution position of the single peak of metalloproteinase (fraction C4a) obtained here, corresponded with that of the second metalloproteinase peak (fraction B4g) obtained previously (Ward 1975*b*).

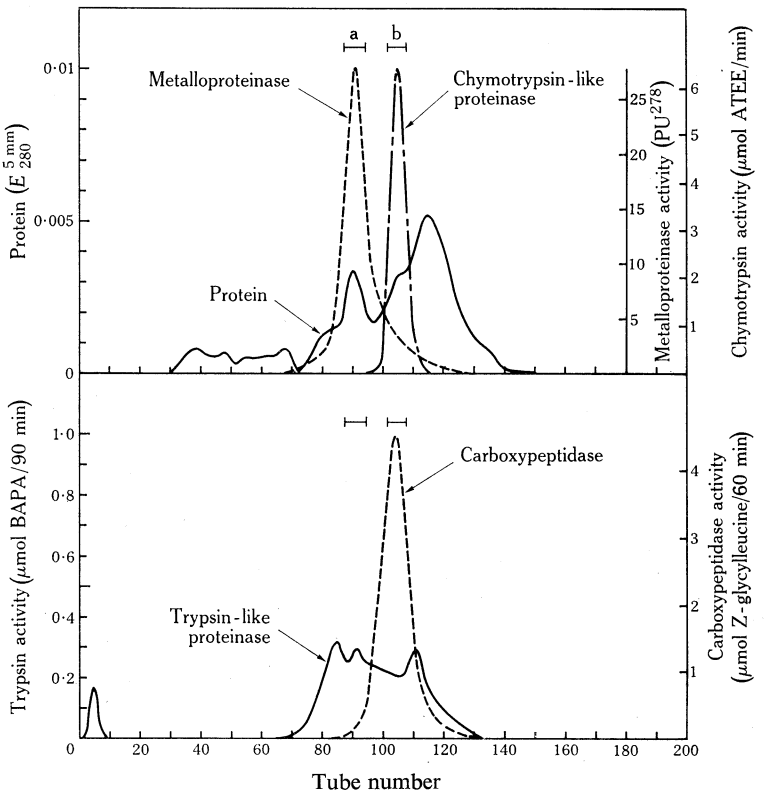


Fig. 1. Chromatography of fraction C4 on DEAE-cellulose column (0·9 × 15 cm). Flow rate was 9·3 ml/h, fraction volumes were 3·1 ml. Sample (6·0 ml) was pumped on followed by 26 ml of starting buffer (0·01M tris–HCl, pH 7·3). At tube 13, a linear gradient was established from 0·01M tris–HCl, pH 7·3 (300 ml), to 0·3M NaCl in the same buffer (300 ml). Every second tube was assayed for metalloproteinase activity, trypsin-like activity, chymotrypsin-like activity and carboxypeptidase activity. Tube contents were pooled to give fractions C4a and C4b. Abbreviations used: BAPA, *N*-benzoyl-DL-arginine *p*-nitroanilide; Z, *N*-benzyloxycarbonyl derivative; ATEE, *N*-acetyl-L-tyrosine ethyl ester. Metalloproteinase units were estimated from a standard curve of enzyme concentration against digestion products as measured by the spectrosphotometric (PU^{278}) procedure.

Acrylamide gel electrophoresis (Fig. 2) further showed that the material in fraction C4a was very similar to that in fraction B4g, consisting essentially of a single protein band with a mobility relative to that of bromphenol blue of 0·52 under the conditions employed.

These results suggest that there are only two metal chelator-sensitive proteinases present in extracts of *T. bisselliella* larvae and for convenience they shall be referred to from now on as metalloproteinase 1 and metalloproteinase 2. Metalloproteinase 1

is that present in fraction B4f and several of its properties have been described previously (Ward 1975*b*). Metalloproteinase 2 is that present in fractions B4g and C4a and some of its properties will now be described. For these experiments the metalloproteinase 2 preparations were always pretreated with DFP (Ward 1975*b*) to inactivate the traces of trypsin-like activity still present.

Properties of Metalloproteinase 2

pH optimum. The effect of pH on the hydrolysis of casein by metalloproteinase 2 is shown in Fig. 3.

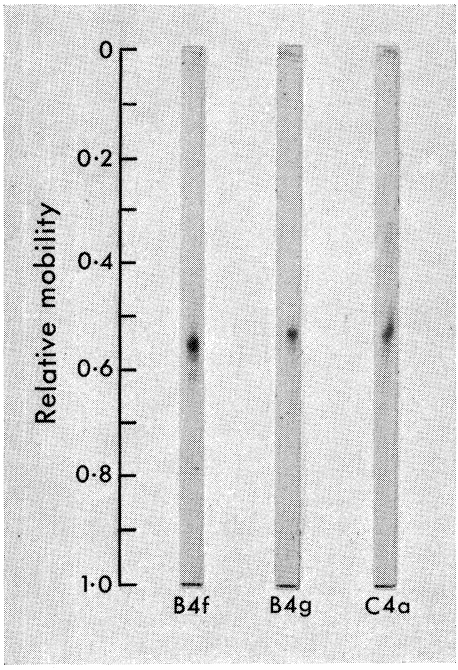


Fig. 2. Comparison of protein bands in fraction C4a with those in fraction B4f and B4g obtained as described by Ward (1975*b*). The running gel contained 7·5% acrylamide, migration is towards the anode and is expressed relative to the mobility of bromphenol blue.

Stability. The effect of pH on the stability of metalloproteinase 2 is shown in Fig. 4. The enzyme was quite stable at alkaline pH with maximum stability between pH 9 and 9·5. The enzyme was unstable at acid pH.

Inhibitors. As shown in Table 1, metalloproteinase 2 is completely inhibited by EDTA, but not by the complex of EDTA and calcium. Thus the inhibition by EDTA is due to its chelating properties. EDTA inhibition can be almost completely reversed by the addition of calcium and zinc ions and partially reversed by the addition of magnesium and of copper ions. The effects of metal cations alone on the activity of metalloproteinase 2 are shown in Table 2. Only mercuric ions produced complete inhibition. Calcium, magnesium and zinc ions produced no inhibition, while copper nickel, chromium and cobalt ions produced partial inhibition.

Specificity of Metalloproteinase 2 in the Cleavage of Insulin Chains

The peptides produced when S-Cm-A-chain insulin was digested with metallo-

proteinase 2 are shown in Fig. 5. Peptide 2 could have been either Leu-Tyr-Gln or Tyr-Gln-Leu from its amino acid composition but *N*-terminal analysis by the Dansyl method (Gray 1967) showed that leucine was the *N*-terminal residue. Thus peptide 2 is Leu-Tyr-Gln and peptide 4 is assumed to be Leu, not Leu.

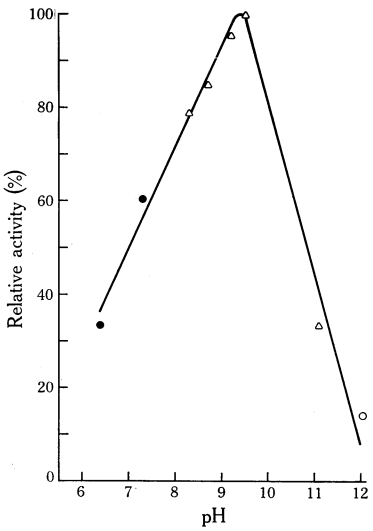


Fig. 3. Effect of pH on metalloproteinase 2 activity expressed as PU^{278}. ● 0·05M sodium phosphate buffer. △ 0·05M Diol buffer. ○ 0·05M KOH.

The peptides obtained when S-Cm-B-chain insulin was digested with metalloproteinase 2 are shown in Fig. 6. From a consideration of the other peptides obtained, peptides 6 (glycine) and 7 (S-Cm-Cys) are assumed to represent residues Gly and Cys respectively, and not residues Gly, Gly or Cys. The data characterizing these peptides from the insulin chains are presented in a supplement.*

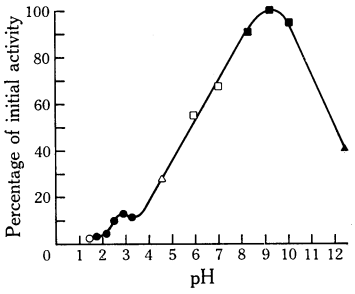


Fig. 4. pH Stability of metalloproteinase 2. The enzyme was held at the pH indicated for 24 h at 4°C, then casein was added and residual proteinase activity determined at pH 9·5 and 37°C. The buffers used at 0·05M were HCl (○), glycine–HCl (●), sodium acetate (△), sodium phosphate (□), Diol buffer (■) and KOH (▲).

* Supplementary data to this paper giving details of the insulin A and B chain peptides are deposited with and copies may be obtained from the Editor-in-Chief, Editorial and Publications Service, CSIRO, 372 Albert Street, East Melbourne, Vic. 3002.

Discussion

The results of this and previous investigations (Ward 1975*a*, 1975*b*) indicate that extracts from *T. bisselliella* larvae contain only two major metal chelator-sensitive proteinases. They are presumably metalloproteinases (Vallee and Wacker 1970) in view of their responses to active site inhibitors and metal cations and have been termed for convenience metalloproteinase 1 and metalloproteinase 2. They are present in approximately equal proportions in *T. bisselliella* larval extracts.

Table 1. EDTA inhibition of metalloproteinase 2 and its reversal by metal ions

The enzyme was pre-incubated at pH 9·4 with 0·0017M EDTA or calcium–EDTA complex for 15 min at 30°C (first treatment). Metal ion was then added (0·0033M final concn) and the incubation continued for a further 15 min (second treatment). The residual proteinase activity was determined at pH 9·4 with casein as described previously (Ward 1975*a*)

First treatment	Second treatment	Relative activity (%)	First treatment	Second treatment	Relative activity (%)
—	—	100	EDTA	$MgCl_2$	24
Ca–EDTA complex	—	92	EDTA	$CuCl_2$	15
EDTA	—	0	EDTA	$CrCl_3$	8
EDTA	$CaCl_2$	80	EDTA	$NiCl_2$	4
EDTA	$ZnCl_2$	90			

Some of the properties of metalloproteinase 1 (from fraction B4f) have been described in an earlier paper (Ward 1975*b*). The present results indicate that metalloproteinase 2 is very similar to metalloproteinase 1. The effect of pH on both enzyme activities and their responses to EDTA, the calcium–EDTA complex and metal

Table 2. Effect of metal ions on metalloproteinase 2 activity

The enzyme was pre-incubated with 0·0017M 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 (Ward 1975*a*)

Addition	Relative activity (%)	Addition	Relative activity (%)
None	100	$NiCl_2$	75
$CaCl_2$	107	$CrCl_3$	62
$MgCl_2$	106	$CoCl_3$	50
$ZnCl_2$	98	$HgCl_2$	0
$CuCl_2$	80		

cations are very similar. In addition their cleavage specificities against S-Cm-insulin A and B chains are almost identical except for several minor cleavages. The enzymes do differ in their electrophoretic mobilities in acrylamide gels, their chromatography on ion-exchange columns and in their stability under various pH conditions. Both enzymes are quite stable at alkaline pH although maximum stability occurs at different pH values (10·3 and 9·3 for metalloproteinases 1 and 2 respectively). In addition metalloproteinase 2 does not show the additional high stability around pH 2·3 as found for metalloproteinase 1 (Ward 1975*b*).

Fig. 5

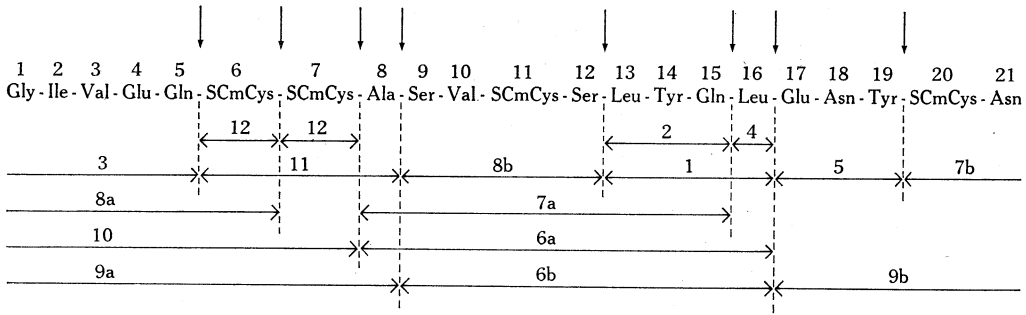
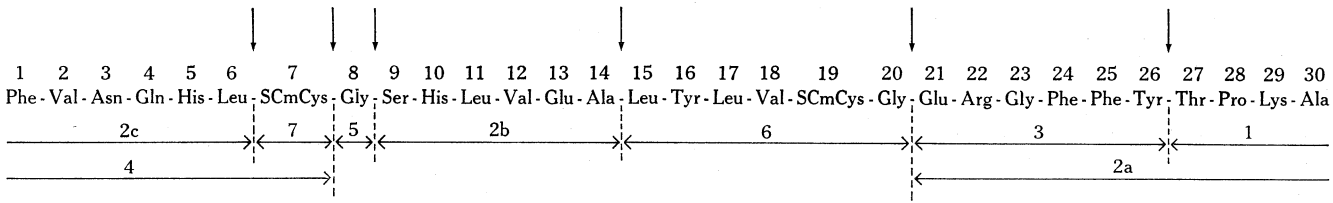


Fig. 6



Figs 5 and 6. Sites of cleavage of S-Cm-A-chain insulin (Fig. 5) and S-Cm-B-chain insulin (Fig. 6) by metalloproteinase 2. Deductions were made from the data in the supplement.

The specificity limitations of these *Tineola* metalloproteinases and the significance of such enzymes in the digestive tract of this insect have been discussed previously (Ward 1975*b*). Whether such metalloproteinases are more widely distributed among insects and other invertebrates which lack pepsin-like proteinases (De Villez 1968) remains to be established. DFP-insensitive alkaline proteinases have now been reported in crayfish gastric juice (De Villez 1968; De Villez and Lau 1970), shrimp hepatopancreas (Gates and Travis 1973) and in extracts of the honey bee (Giebel *et al.* 1971), but it has not been established whether these proteinases are metal chelator-sensitive and are true metalloproteinases. Metalloproteinases have been reported recently in the moulting fluid of the tobacco hornworm (Bade and Shoukimas 1973) and in extracts of the pyloric caeca of the starfish, *Lysastrosoma anthosticta* (Elyakova and Kozlovskaya 1975; Kozlovskaya and Elyakova 1975).

Acknowledgments

The author wishes to thank Mr W. J. Youl for his technical assistance, Mr K. H. Gough for the dansyl end group determinations and Dr C. M. Roxburgh for the amino acid analyses.

References

Bade, M. L., and Shoukimas, J. J. (1973). *Fed. Proc.* **32**, 503.
De Villez, E. J. (1968). *Am. Zool.* **8**, 541.
De Villez, E. J., and Lau, J. (1970). *Int. J. Biochem.* **1**, 108–14.
Elyakova, L. A., and Kozlovskaya, E. P. (1975). *Comp. Biochem. Physiol.* **50B**, 249–53.
Gates, B. J., and Travis, J. (1973). *Diss. Abstr.* **33**, 4106–B.
Giebel, W., Zwilling, R., and Pfleiderer, G. (1971). *Comp. Biochem. Physiol.* **38B**, 197–210.
Gray, W. R. (1967). *Methods Enzymol.* **11**, 139–51.
Kozlovskaya, E. P., and Elyakova, L. A. (1975). *Comp. Biochem. Physiol.* **50B**, 255–8.
Vallee, B. L., and Wacker, W. E. C. (1970). In 'The Proteins'. (Ed. H. Neurath.) 2nd Ed. Vol. 5, pp. 129–46. (Academic Press: New York.)
Ward, C. W. (1975*a*). *Aust. J. Biol. Sci.* **28**, 1–23.
Ward, C. W. (1975*b*). *Biochem. Biophys. Acta* **384**, 215–17.

Manuscript received 8 July 1975