Biochimica et Biophysica Acta, 391 (1975) 201—211

BBA 67481

PROPERTIES AND SPECIFICITY OF THE MAJOR ANIONIC TRYPSIN-LIKE ENZYME IN THE KERATINOLYTIC LARVAE OF THE WEBBING CLOTHES MOTH

COLIN W. WARD

Division of Protein Chemistry, CSIRO, Parkville (Melbourne), Victoria 3052 (Australia)

(Received October 25th, 1974)

Summary

The major form of the trypsin-like proteinases from the larvae of the webbing clothes moth *Tineola bisselliella* has been further purified and some of its properties investigated. It differs from bovine trypsin in several respects. It is anionic at neutral pH, is very stable at alkaline pH, has no requirement for calcium ions for this stability and is very sensitive to urea. It resembles vertebrate trypsins in its complete inhibition by diisopropylfluorophosphate, its pH optimum of 8.5 for hydrolysis of benzoyl-arginine *p*-nitroanilide and its cleavage specificity against glucagon and the β-chain of *S*-carboxymethyl insulin.

Introduction

Earlier studies on the proteinases of *Tineola bisselliella* larvae demonstrated the presence of considerable trypsin-like activity in this organism [1]. Both cationic forms (1 band) and anionic forms (7 bands) could be detected on acrylamide gel zymograms and all forms appeared to be associated with the digestive tract. These enzymes were trypsin-like because of their complete inhibition of Dip-F [2] and their ability to hydrolyse the chromogenic trypsin substrates Bz-Arg-NapN [3,4] and Bz-Arg-NAn [5]. However these two criteria do not guarantee that these enzymes are analogous [6] with vertebrate trypsins in their cleavage specificity or other properties. The "trypsin" from the insect *Tenebrio molitor*, although hydrolysing benzoyl-arginine ethyl ester and polylysine and being inhibited by several natural protein trypsin inhibitors dis-

Abbreviations: Ac-Tyr, *N*-acetyl-DL-tyrosine; Ac-Tyr-OEt, *N*-acetyl-L-tyrosine ethyl ester; Bz-Arg-OEt, *N*-benzoyl-L-arginine 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; Tosyl-Arg-OMe, α-*N*-toluene sulfonyl-L-arginine methyl ester; Z-Gly-Leu, carbobenzoxy-glycyl-leucine.

played completely non-tryptic cleavage specificity with protein substrates [7]. Similarly the Dip-F-sensitive, Bz-Arg-OEt-hydrolysing enzyme from cotton seeds was shown to be a carboxypeptidase and not a trypsin-like enzyme [8]. As Zwilling et al. [7] point out, caution must be used in referring to enzymes as trypsin-like, before their cleavage specificity on protein substrates has been determined. Enzymes showing typical trypsin cleavage specificity have been denemonstrated in the insects *Apis mellifica* [9], *Vespa orientalis* [10], *Locusta migratoria* [11] and *Manduca sexta* [12].

In this paper the major anionic Dip-F-sensitive, Bz-Arg-NAn hydrolysing activity from *T. bisselliella* larvae has been purified further and some of its properties, including its cleavage specificity against glucagon and the β-chain of S-Cm-β-insulin, are described.

Materials and Methods

Chemicals

Chemicals used and their sources were: Ac-Tyr, Ac-Tyr-OEt, Bz-Arg-NAn, Z-gly-leu and glucagon, Schwartz-Mann Research Laboratories; Bz-Arg-NapN, Calbiochem; Dip-F, Aldrich Chemical Co.; bovine serum albumin, Sigma Chemical Co.; fast garnet G.B.C., G.T. Gurr Ltd, England; Hammarsten casein, E. Merck, Germany; DEAE-cellulose (DE-52), Reeve Angel & Co., England. The *S*-carboxymethyl β-chain of bovine insulin was a gift from I.J. O'Donnell of this laboratory.

Enzyme

The starting material used for this study was fraction C3, obtained and purified as described previously [1] and stored in 50% glycerol at −20° C until used.

Enzyme assays, column chromatography, ultrafiltration and acrylamide gel electrophoresis

These were carried out as described previously in ref. 1.

Enzyme kinetic determinations

The kinetic parameters K_m and V for the hydrolysis of DL-Bz-Arg-NAn were determined by the method of Lee and Wilson [13] where the instantaneous velocity is replaced by the average velocity over a fixed time period and the initial substrate concentration is replaced by the arithmetic average substrate concentration during that time interval. Regression analysis of the Lineweaver-Burk plots:

$$\frac{1}{\bar{v}} = \frac{1}{\bar{S}} \cdot \frac{K_m}{V} + \frac{1}{V} \quad \text{and}$$

$$\frac{\bar{S}}{\bar{v}} = \frac{1}{V} \cdot \bar{S} + \frac{K_m}{V} \quad \text{and}$$

the Eadie-Hofstee plots:

$$\frac{\bar{v}}{\bar{S}} = \frac{-1}{K_m}\bar{v} + \frac{V}{K_m}$$

were carried out on a GE terminal computer. The K_m and V values from these three regression analyses were then averaged.

Protein determination

Protein determination was by the method of Lowry et al. [14] with bovine serum albumin as standard.

Cleavage specificity determinations

Digestion. Before adding to the digestion mixture the trypsin-like proteinase was pre-treated with EDTA to inhibit the residual metal-chelator sensitive protease present. The pre-treatment reaction mixture (0.2 ml) contained: Diol buffer, pH 8.5, 1.0 μmol; EDTA 2.0 μmol and protease, 7 μg. This was held at 5° C for 60 min and then added to a solution (0.8 ml) containing Diol buffer, pH 8.5 and either S-Cm-β-insulin, 5.0 mg or glucagon 5.0 mg, and incubated at 37° C for 4.0 h (β-insulin) or 6.0 h (glucagon).

Analysis of peptides and amino acid analyses. These were carried out as described previously [15]. Tryptophan-containing peptides were identified by spraying the chromatograms with 1% (w/v) 4-dimethylaminobenzaldehyde in acetone/conc. HCl (9/1) and were hydrolysed, in the presence of 1 mg tryptamine, with 0.5 ml 3 M *p*-toluene sulphonic acid for 24 h at 110° C in sealed and evacuated tubes.

Results

Final purification

The starting material was fraction C3, obtained and purified as previously described [1]. This fraction, although not containing as much trypsin-like activity as fractions C4 and B4, was more homogeneous than these fractions with respect to the various forms of trypsin-like activity present. As shown in Fig. 1, C3 contained essentially a single trypsin-like band, (R_F 0.53), this being the major anionic trypsin-like enzyme found in *T. bisselliella* larvae. Both C4 and B4 were heavily contaminated with the other bands, R_F 0.60, 0.65 and 0.72. Further, unlike C4 and B4, fraction C3 contained none of the cationic trypsin-like activity. C3 did contain carboxypeptidase activity and small amounts of chymotrypsin-like proteinase and metallo proteinase activities but no aminopeptidase activity [1].

The fractionation of C3 by downward flow gel filtration on two columns (each 1.5 cm × 100 cm, connected in series) of Sephadex G-100 is shown in Fig. 2. A single peak of trypsin-like activity was obtained which was well separated from the bulk of the contaminating protein and the carboxypeptidase activity, but only partially separated from the metal-chelator sensitive proteinase and the chymotrypsin-like enzyme in the original material. The tubes containing most of the trypsin-like activity (C3b) were pooled, concentrated by ultrafiltration and stored in 50% glycerol at −20° C.

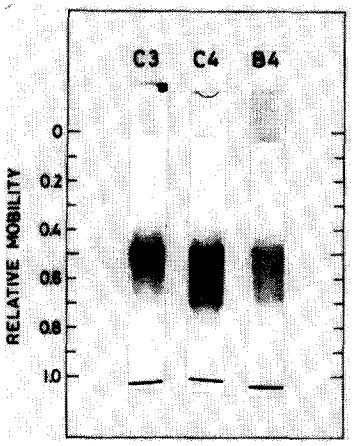

Fig. 1. Anionic trypsin-like bands in fractions C3, C4 and B4. Electrophoresis in 7.5% running gels was as described previously [1]. Enzyme bands were detected by incubating the gels at pH 9.8 with 0.5 mM Bz-Arg-NapN for 30 min at room temperature and staining with 0.15% fast garnet GBC in acetate-detergent buffer, pH 4.5 as previously described [1]. Migration is towards the anode and mobility is expressed relative to that of bromephenol blue.

The specific activity of C3b against Bz-Arg-NAn was 17.0 μmol/30 min/mg protein which represents approximately a 14-fold purification over C3 and a 99-fold purification over the original ammonium sulphate fraction which contained multiple forms of trypsin-like activity. The amount of chymotrypsin-like activity in C3b was very low, but the amount of metal-chelator sensitive

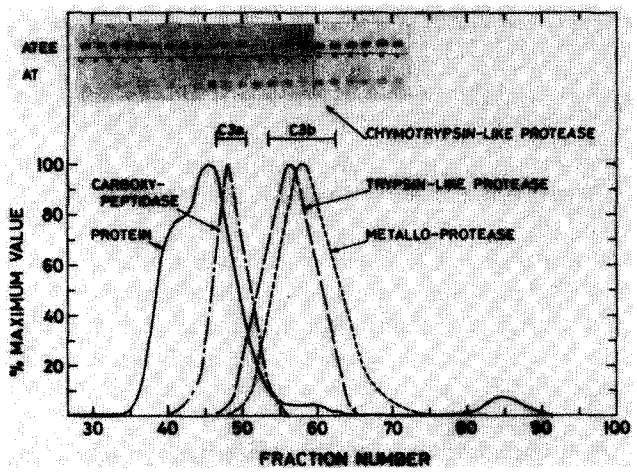

Fig. 2. Gel filtration of fraction C3 on Sephadex G-100 (two columns connected in series, each 1.5 cm × 100 cm). Downward flow rate was 13.9 ml/h maintained by peristaltic pump, fraction volumes were 3.5 ml and the buffer used was 0.1 M Tris · Cl pH 7.5. Sample volume loaded was 3.1 ml. Starting with tube 10 every second tube was assayed for enzyme activity, the results being expressed as a percentage of the maximum activity per tube obtained during the fractionation. These maximum values were: metal-chelator sensitive proteinase, 5.04 $[PU]^{278}$ (ref. 1); trypsin-like activity 1.2 μmol-Bz-Arg-NAn/90 min; carboxypeptidase activity, 10.3 μmol Z-gly-leu/60 min. The chymotrypsin-like activity was not quantitated but was monitored by the high voltage electrophoresis spot test [1]. Fractions C3a and C3b were pooled as shown and concentrated by ultrafiltration.

proteinase in this fraction was significant. Thus in all experiments not involving hydrolysis of the specific substrate Bz-Arg-NAn this metal-chelator sensitive contaminant was inhibited with EDTA [1].

Properties of the trypsin-like proteinase C3b

pH optimum

The effect of pH on the hydrolysis of Bz-Arg-NAn by C3b is shown in Fig. 3. The pH optimum was pH 8.5 and there was negligible activity below pH 6.0.

Stability

Effect of Ca^{2+}. T. bisselliella trypsin is very stable to prolonged incubation at its pH optimum and does not require Ca^{2+} for this stability. When samples of fraction C3b were incubated for periods up to 4 h at pH 8.5 and 37° C in both the presence and absence of $2 \cdot 10^{-3}$ M $CaCl_2$, the loss in activity in both groups of samples was less than 5%.

Effect of urea. As shown in Fig. 4, the *T. bisselliella* trypsin was quite sensitive to increasing concentrations of urea, and was almost completely inactived when the urea concentration in the pre-incubation and assay mixtures reached 4.0 M.

Kinetic parameters for Bz-Arg-NAn hydrolysis

The effect of increasing substrate concentration on enzyme activity is shown in Fig. 5 and the resulting Lineweaver-Burk and Eadie-Hofstee plots

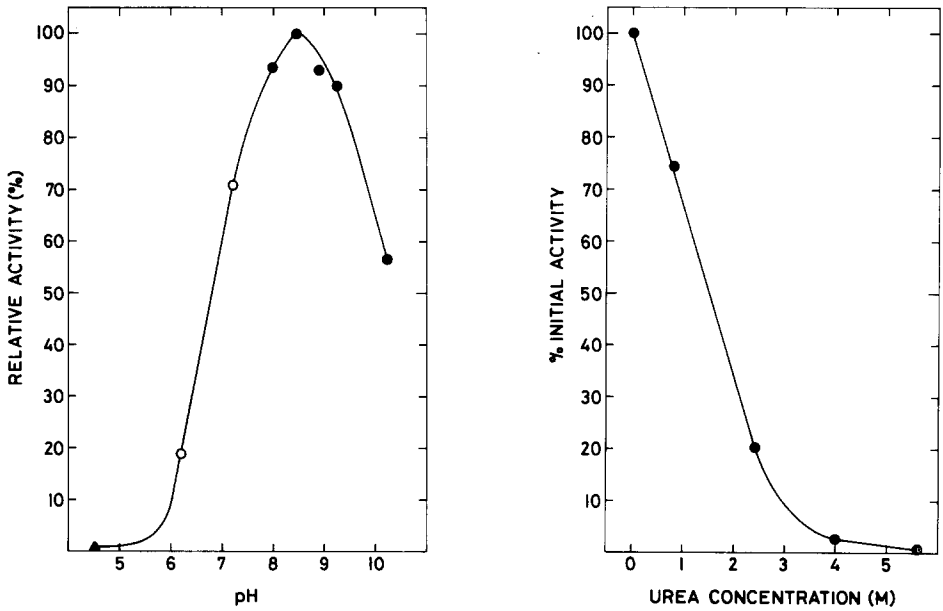

Fig. 3. Effect of pH on Bz-Arg-NAn hydrolysis by fraction C3b. Buffers used at 0.04 M were; sodium acetate/acetic acid; ▲; NaH_2PO_4/NaP_2HPO_4, ○; Diol/HCl buffer, ●. Maximum activity was 17 μmol Bz-Arg-NAn hydrolysed/30 min/mg C3b protein.

Fig. 4. Effect of urea on C3b trypsin-like enzyme. The reaction mixtures (0.5 ml) contained: Diol buffer pH 8.5, 20 μmol; C3b proteinase and urea. They were pre-incubated for 30 min at 20° C and the residual activity assayed on addition of 0.2 μmol of Bz-Arg-NAn.

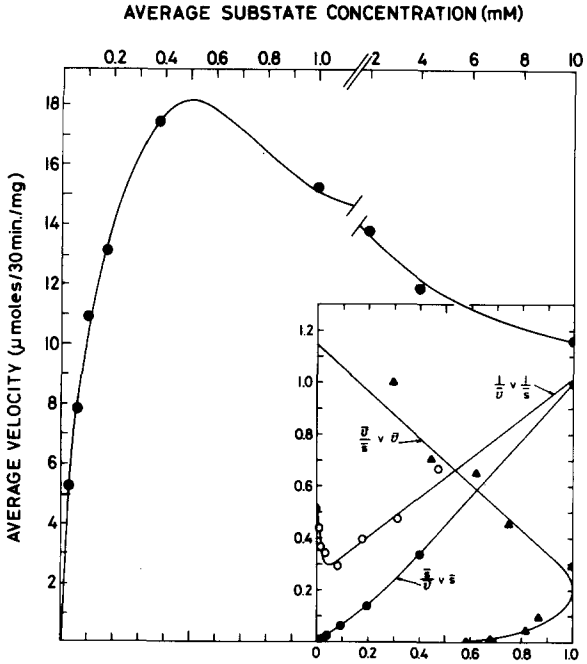

Fig. 5. Effect of substrate concentration on reaction velocity. Here the ordinate is the average velocity over the 30 min incubation period and the abcissa is the average substrate concentration over the same period (see ref. 13). Substrate is DL-Bz-Arg-NAn; temperature, 37° C, pH 8.5; initial substrate concentrations were $0.04 \cdot 10^{-3}$, $0.08 \cdot 10^{-3}$, $0.12 \cdot 10^{-3}$, $0.20 \cdot 10^{-3}$, $0.40 \cdot 10^{-3}$, $1.0 \cdot 10^{-3}$, $2.0 \cdot 10^{-3}$, $4.0 \cdot 10^{-3}$ and $10.0 \cdot 10^{-3}$ M. Insert. Lineweaver-Burk and Eadie-Hofstee plots of kinetic data. S is in mM and v is in μmol Bz-Arg-NAn per 30 min per mg C3b enzyme. The equivalent unit values used in this figure are: ordinate, 0.193 for $\frac{1}{v}$, 0.991 for $\frac{\bar{S}}{v}$ and 159 for $\frac{\bar{v}}{\bar{S}}$; abscissa, 30.72 for $\frac{1}{\bar{S}}$, 9.986 for $\bar{S}$ and 17.4 for $\bar{v}$.

shown in the insert to Fig. 5. As can be seen from both figures the *T. bisselliella* trypsin was inhibited by substrate concentrations above $0.4 \cdot 10^{-3}$ M. The regression analyses for the reciprocal plots were confined to the data in the 0—$0.4 \cdot 10^{-3}$ M substrate range. From these data the K_m (app) was (0.102 ± 0.01) $\cdot 10^{-3}$ M and the V was 21.7 ± 1.16 μmol/30 min/mg C3b.

C3b cleavage specificity of S-Cm-β-insulin chain and glucagon

The amino acid composition, the electrophoretic mobilities, the isolation procedures and the inferred sequences of the peptides obtained following digestion of the S-Cm-β-chain of insulin are summarized in Table I and the location of these peptides in the amino acid sequence of insulin β-chain is shown in Fig. 6. Peptide 4 did not move at either pH 6.5 or pH 3.5 but remained at the origin. Its amino acid analysis is consistent with it being a mixture of two peptides, the first being undigested insulin β-chain and the second being the large peptide Phe_1-Arg_{22}. Peptide 2, residues Gly_{23}-Ala_{30} was the peptide recovered in highest amount but the existence of peptide 3 suggests some cleavage at the Lys_{29}-Ala_{30} bond. However no peptide was found containing just residues Gly_{23}-Lys_{29}.

Peptide 1 indicates a small amount of non-tryptic cleavage at Tyr_{26}-

TABLE I

C3b TRYPSIN-LIKE PROTEINASE PEPTIDES FROM INSULIN S-Cm-β CHAIN

Amino acid	Peptides (residues/molecule)			
	1	2	3	4
Lys	1.0 (1)	0.98 (1)		1.14 (1)
His			0.10	4.17 (4)
Arg				2.06 (2)
S-Cm-Cys				3.42 (4)
Asp	0.16	0.02	0.14	2.23 (2)
Thr	0.62 (1)	1.03 (1)		1.00 (1)
Ser	0.39	0.03	0.15	2.03 (2)
Glu	0.39	0.04	0.25	6.29 (6)
Pro	0.96 (1)	1.04 (1)		1.07 (1)
Gly	1.0	0.96 (1)	0.38	4.97 (5)
Ala	1.42 (1)	0.88 (1)	1.00 (1)	2.97 (3)
Val			0.23	6.26 (6)
Leu			0.32	8.08 (8)
Tyr		1.00 (1)		2.67 (3)
Phe		2.10 (2)		3.66 (4)
m	− 0.47	− 0.304	− 0.02	insoluble
Residues	27–30	23–30	30	1–30 and 1–22
Amount (nmol)	10.8	146	67.6	59.6 for both
% Yield	7.4	100	46.3	40.8
Isolation methods *	3.5	3.5	3.5	3.5

* High-voltage electrophoresis at pH 3.5.

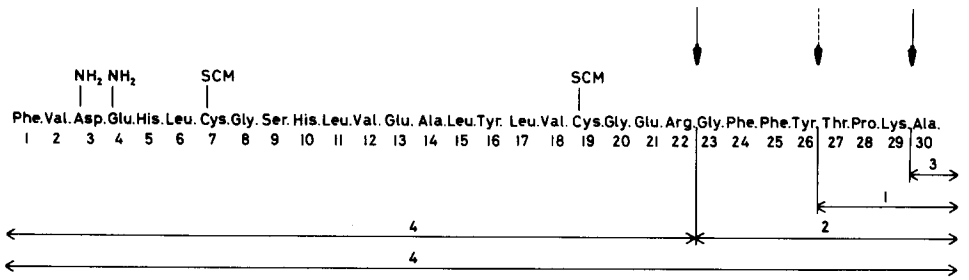

Fig. 6. Sites of cleavage of S-Cm-β-chain insulin by the trypsin-like proteinase (C3b) from *T. bisselliella*. Deductions were made from the data in Table I.

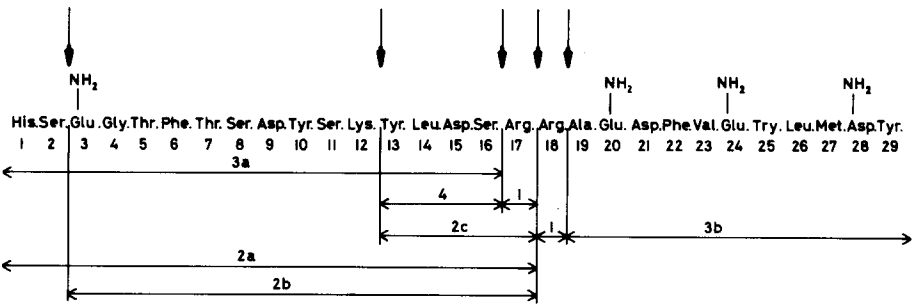

Fig. 7. Sites of cleavage of glucagon by the trypsin-like proteinase (C3b) from *T. bisselliella*. Deductions were made from the data in Table II.

TABLE II

C3b TRYPSIN-LIKE PROTEINASE PEPTIDES FROM GLUCAGON

Amino acid	Peptide (residues/molecule)						
	1	2a	2b	2c	3a	3b	4
Lys	0.09	1.15 (1)	1.12 (1)	0.24	0.96 (1)		
His	0.05	0.69 (1)	0.24		0.54 (1)		
Arg	1.00 (1)	0.75 (1)	0.97 (1)	0.82 (1)			
Asp		2.00 (2)	2.20 (2)	1.10 (1)	2.26 (2)	2.33 (2)	1.47 (1)
Thr		2.38 (2)	1.66 (2)	0.37	1.98 (2)	1.26 (1)	0.23
Ser		3.81 (4)	3.22 (3)	1.29 (1)	3.78 (4)	0.41	0.92 (1)
Glu		1.18 (1)	1.22 (1)	0.18	1.13 (1)	2.40 (2)	0.50
Gly		1.39 (1)	0.98 (1)	0.26	1.47 (1)	0.50	0.22
Ala			0.48			0.97 (1)	0.25
Val						0.93 (1)	0.17
Met						0.85 (1)	
Leu		0.76 (1)	0.95 (1)	0.87 (1)	1.44 (1)	1.15 (1)	0.96 (1)
Tyr		1.74 (2)	1.47 (2)	0.91 (1)	1.75 (2)		0.64 (1)
Phe		1.15 (1)	1.23 (1)	0.20	1.24 (1)	1.11 (1)	0.39
Tryp						0.88 (1)	
m	– 0.95	0.00	0.00	0.00	+ 0.20	+ 0.20	+ 0.45
Residues	17.18	1–17	3–17	13–17	1–16	19–29	13–16
Amount (nmol)	196.8	62.2	55.2	88.6	21.3	25.8	64.2
% Yield	100	31.6	28.0	45.0	10.8	13.1	32.6
Isolation	6.5	6.5	6.5	6.5	6.5	6.5	6.5
Methods *		3.5	3.5	3.5	3.5	3.5	

* High voltage electrophoresis at pH 3.5 and/or pH 6.5

Thr_{27}, which could be produced by the C3b trypsin or by either the chymotrypsin-like or metallo-protease contaminants.

The data characterising the peptides obtained following C3b digestion of glucagon is summarized in Table II and the location of these peptides in the amino acid sequence of glucagon is shown in Fig. 7. Typical tryptic cleavages occurred at Lys_{12}-Tyr_{13}, Arg_{17}-Arg_{18} and Arg_{18}-Ala_{19} but additional non-tryptic cleavages occurred at Ser_2-Gln_3 and Ser_{16}-Arg_{17}.

Discussion

In a previous study [1] extracts of *T. bisselliella* larvae were shown to contain several Dip-F-sensitive, Bz-Arg-NapN- and Bz-Arg-NAn-hydrolysing activities. The enzymes were termed trypsin-like proteinases although these two criteria do not guarantee that the enzymes are truly analogous to trypsin. Other proteinases such as some cathepsins [16,17] and the plant thiol proteinases [18—20] can readily hydrolyse Bz-Arg-NapN and Bz-Arg-NAn and some "trypsin-like" enzymes have been found to be completely unlike trypsin when their cleavage specificities on protein substrates has been investigated [7,8].

Earlier results [1] showed that the trypsin-like enzymes from *T. bisselliella* differed from bovine trypsin in several respects. They had low isoelectric points (with one exception), were unstable at acid pH, but very stable under alkaline conditions, and were unaffected by the natural trypsin inhibitors ovomucoid, soybean trypsin inhibitor and lima bean trypsin inhibitor. In this report the major anionic form of these trypsin-like enzymes in *T. bisselliella* larvae has been purified further and some additional properties compared with those of other trypsins.

In contrast to the situation with vertebrate trypsins [21], Ca^{2+} had no effect on *T. bisselliella* trypsin activity or stability, the enzyme being stable for several hours in the absence of Ca^{2+} at 37° C. Similar findings have been reported for shrimp trypsin [22] and the trypsins from three species of starfish [23—25].

T. bisselliella trypsin further differed from vertebrate trypsins in its sensitivity to urea. Bovine trypsin was still quite active in 4 M urea [26] whereas *T. bisselliella* trypsin had lost almost all of its activity under similar conditions. In this respect it was very similar to the special trypsin-like proteinase, cocoonase [27]. The sensitivity of this latter enzyme to urea was correlated with the presence of only a single disulphide bond compared to six such bonds in vertebrate trypsin.

The pH optimum for Bz-Arg-NAn hydrolysis by *T. bisselliella* trypsin was 8.5 which was very similar to those obtained for the hydrolysis of this substrate by vertebrate [5] and invertebrate [24,25] trypsins and for the hydrolysis of Bz-Arg-OEt, Tosyl-Arg-OMe and casein by several other invertebrate trypsins [10,22,23,28,29].

T. bisselliella trypsin has a high affinity for Bz-Arg-NAn. The K_m value was $0.1 \cdot 10^{-3}$ M which is higher than the values of $0.014 \cdot 10^{-3}$ $0.039 \cdot 10^{-3}$ and $0.065 \cdot 10^{-3}$ M obtained for the trypsin-like enzymes of three species of *Streptomyces* [30], but lower than the values of $0.94 \cdot 10^{-3}$ M [5] and $1.49 \cdot 10^{-3}$ M [30] obtained for bovine trypsin. At substrate concentrations above

$0.4 \cdot 10^{-3}$ M, substrate inhibition became quite apparent, due presumably to the presence of D-Bz-Arg-NAn in the DL-substrate mixture employed [5].

The data obtained for the digestion of glucagon and S-Cm-insulin β-chain indicate that the trypsin-like enzyme from *T. bisselliella* larvae is functionally analogous to other trypsins. Major splits occurred at the Arg_{22}-Gly_{23} and Lys_{29}-Ala_{30} bonds in insulin β-chain (Fig. 6) and at the Lys_{12}-Tyr_{13}, Arg_{17}-Arg_{18} and Arg_{18}-Ala_{19} bonds in glucagon (Fig. 7) and these were the only bonds in these substrates split by vertebrate trypsins. However additional non-tryptic cleavages occurred which may have resulted from either a broader specificity of the *T. bisselliella* trypsin or from some residual activity of the chymotrypsin-like or EDTA inhibited metalloproteinase contaminants present in the C3b preparation. These residual activities were negligible as judged by normal assay procedures, but may have produced some significant cleavage during the long incubation periods (4—6 h) used for the specificity studies. With insulin β-chain only one non-tryptic peptide was obtained and was present in very low amount (7%). This peptide, Thr_{27}-Ala_{30}, was one of the major peptides produced during the digestion of insulin β-chain with *T. bisselliella* metal-chelator sensitive proteinases [15] and its presence in the C3b digests was probably due to some residual activity of the EDTA treated metallo-proteinase contaminant. With glucagon, non-tryptic cleavages occurred at the Ser_2-Gln_3 and Ser_{16}-Arg_{17} bonds. It is interesting to note that one of the trypsins (protease A) from the starfish *Dermesterias imbricata* gave additional non-tryptic cleavages at the Ser_{16}-Arg_{17} and the Ser_{11}-Lys_{12} bonds of glucagon [24] although the authors suggested that a contaminating carboxypeptidase may have produced these cleavages. An additional minor non-tryptic peptide was also produced from glucagon by the trypsin-like enzyme from Tobacco hornworm larvae although the chemical composition of the peptide was not investigated [12].

The results in this paper demonstrate that the major anionic Bz-Arg-NAn-hydrolysing enzyme for *T. bisselliella* larvae is a true trypsin-like enzyme. Though differing from bovine trypsin in some of its properties such as its isoelectric point and its stability characteristics, it is a serine protease, with an alkaline pH optima and a cleavage specificity identical to or slightly broader than that of vertebrate trypsins.

Acknowledgements

I wish to thank Mr W.J. Youl for his technical assistance and Dr C.M. Roxburgh for the amino acid analyses.

References

1 Ward, C.W. (1975) Aust. J. Biol. Sci. 28, 1—23
2 Hartley, B.S. (1960) Annu. Rev. Biochem. 29, 45—72
3 Riedel, A. and Wuensch, E. (1959) Hoppe-Seyler's Z. Physiol. Chem. 316, 61—70
4 Blackwood, C. and Mandl, I. (1961) Anal. Biochem. 2, 370—379
5 Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) Arch. Biochem. Biophys. 95, 271—278
6 Neurath, H., Walsh, K.A. and Winter, W.P. (1967) Science 158, 1638—1644
7 Zwilling, R., Medugorac, I. and Mella, K. (1972) Comp. Biochem. Physiol. 43B, 419—424

8 Ihle, J.N. and Dure, L.S. (1972) J. Biol. Chem. 247, 5041—5047
9 Giebel, W., Zwilling, R. and Pfleiderer, G. (1971) Comp. Biochem. Physiol. 38B, 197—210
10 Hagenmaier, H.E. (1971) J. Insect Physiol. 17, 1995—2004
11 Knecht, M., Hagenmaier, K.E. and Zebe, B. (1974) J. Insect Physiol. 20, 461—470
12 Miller, J.W., Kramer, K.J. and Law, J.H. (1974) Comp. Biochem. Physiol. 48B, 117—129
13 Lee, H.-J. and Wilson, I.B. (1971) Biochim. Biophys. Acta 242, 519—522
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Ward, C.W. (1975) Biochim. Biophys. Acta 384, 215—227
16 Barrett, A.J. (1972) Anal. Biochem. 47, 280—293
17 Distelmair, P., Hubner, H. and Otto, K. (1972) Enzymologia 42, 363—375
18 Ota, S., Moore, S. and Stein, W.H. (1964) Biochemistry 3, 180—185
19 Arnon, R. (1965) Immunochemistry 2, 107—114
20 Englund, P.T., King, T.P., Craig, L.C. and Walti, A. (1968) Biochemistry 7, 163—175
21 Buck, F.F., Vithayathil, A.J., Bier, M. and Nord, F.F. (1962) Arch. Biochem. Biophys. 97, 417—424
22 Gates, B.J. and Travis, J. (1969) Biochemistry 8, 4483—4489
23 Winter, W.P. and Neurath, H. (1970) Biochemistry 9, 4673—4679
24 Camacho, Z., Brown, J.R. and Kitto, G.B. (1970) J. Biol. Chem. 245, 3964—3972
25 Bundy, H.F. and Gustafson, J. (1973) Comp. Biochem. Physiol. 44B, 241—251
26 Harris, J.I. (1956) Nature 177, 471—473
27 Kafatos, F.C., Tartakoff, A.M. and Law, J.H. (1967) J. Biol. Chem. 242, 1477—1487
28 Yang, Y.J. and Davies, D.M. (1968) J. Insect Physiol. 14, 205—222
29 Peng, R.K.Y. and Williams, D.C. (1973) Comp. Biochem. Physiol. 44B, 1207—1217
30 Yoshida, N., Sasaki, A. and Inoue, H. (1971) FEBS Lett. 15, 129—132