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Some Properties of the Extracellular Proteinase and the Cell-bound Peptidase of *Serratia*

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The formation of an extracellular proteinase has been studied in a strain of *Serratia*. The enzyme has a relatively low molecular weight and is truly extracellular because it is formed during exponential growth and no enzyme is associated with the cells. Its formation is repressed by glucose. Other proteolytic enzymes may appear in the medium as a result of autolysis.

Several peptidases have been detected in cell extracts which also contain a proteinase of high molecular weight. The peptidases require divalent metal ions and are also present in cells grown in synthetic media without proteins or peptones. One of them is of the leucine aminopeptidase type, another cleaves glycyl peptides rapidly and two different enzymes hydrolyze glycyl-L-proline and L-alanyl-L-proline, respectively.

Typical strains of the genus *Serratia* form prodigiosin pigments which give them a characteristic red or yellow colour. They are also strongly proteolytic and this is another important taxonomic property. The influence of culture conditions on the formation of proteolytic enzymes in culture filtrates of various *Serratia* strains has been studied earlier by other authors,[1–3] but little is known about the cell-bound proteolytic enzymes of this organism. It was shown in an earlier report from this laboratory [4] that the cells contain an aminopeptidase which hydrolyzes L-leucine amide. Cell extracts also contain a different enzyme which cleaves amino acid naphthyl amides, which are often used in routine determinations of leucine aminopeptidase activity.

This report shows that *Serratia* secretes a true extracellular proteinase but that cell-bound enzymes may be liberated into the medium as a result of autolysis. Fractionation experiments with cell extracts have also been carried out, and they demonstrate that the cells contain a variety of peptidases.

MATERIAL AND METHODS

Bacterial strains and culture conditions. Most experiments were carried out with the same organism and under similar conditions as described earlier.[4] Some comparative growth studies were also made with the Nima strain of the type species *Serratia marcescens*,

and these gave essentially similar results as *Serratia indica*, which was used for the fractionation work.

The following basal medium (M 63) was used: KH_2PO_4 13.6 g, $(NH_4)_2SO_4$ 2 g, $FeSO_4 \cdot 7H_2O$ 0.5 mg, $MgSO_4 \cdot 7H_2O$ 0.1 g, $CaCl_2 \cdot 6H_2O$ 10 mg; distilled water 1 l; pH was adjusted to 7. A carbon source was added as described in the text.

Enzyme assays. We found that 0.01 % merthiolate could be added to culture samples and enzyme solutions to prevent bacterial growth, without lowering the caseinolytic activity. 1 ml of enzyme solution was incubated at 37° with 1 ml of a 1 % solution of casein (Merck) in 0.1 M Tris adjusted to pH 8 with HCl. The reaction was terminated, usually after 60 min, by the addition of 1 ml of 5 % trichloroacetic acid (TCA). After filtration, the absorbance of the filtrate was read at 280 mμ against a blank to which TCA was added before the enzyme solution.

The hydrolysis of peptides was followed qualitatively by thin layer chromatography as described earlier.[5] Determinations of peptidase activities were performed at 37° with the ninhydrin procedure of Matheson and Tattrie [6] at a substrate concentration of 0.1 % in Tris-HCl buffer, pH 8. The release of free proline from proline-containing dipeptides was followed with the method of Sarid *et al.*[7] under similar conditions as above. Enzyme concentration and incubation times were chosen to give as good kinetically valid data as possible, but no attempts were made in these studies to find optimal assay conditions.

Peptides were obtained from Nutritional Biochemicals Corp., USA, and Yeda Inc., Israel. Their names from now on are abbreviated in the conventional way, *e.g.* leu-gly for L-leucyl-glycine.

Fraction experiments. The same methods as described earlier [4] were used. In addition the new method of Lewis and Harris [8] for characterizing peptidases by starch gel electrophoresis was tried.

RESULTS

1. *Extracellular enzymes.* Castañeda-Agulló [2] has shown that *Serratia* forms appreciable amounts of proteinase only when grown in media containing proteins on peptones and that very little enzyme is formed in synthetic media containing sugars. This was confirmed by us. The reason for this could be that the enzyme is inducible, but the low rate of proteinase formation in media containing carbohydrates may also be due to catabolic repression. Fig. 1 illustrates that the addition of glucose to a *Serratia* culture growing in a medium containing gelatine and peptone has a strong repressive effect on

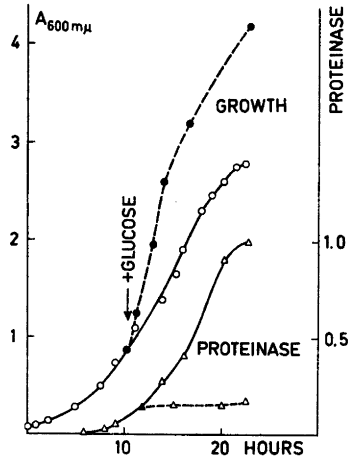

Fig. 1. Extracellular proteinase formation by *Serratia indica* grown in medium 63 with 1 % gelatine and 0.1 % peptone. Growth was estimated photometrically at 600 mμ and the caseinolytic activity was determined on cell-free culture samples. The culture was divided at the time indicated by the arrow, and 0.5 % glucose was added to one part of it (— — — —).

the proteinase formation. In contrast, both the rate of growth and the growth yield were considerably increased by the glucose addition. Similar effects were obtained with other carbohydrates such as fructose and lactose which are readily utilized by *Serratia*.

Furthermore, Fig. 1 shows that the proteinase is truly extracellular, and that it is not only liberated from the cells as a result of autolysis. After an initial lag period, the appearance of extracellular caseinolytic activity paralleled the growth curve. The activities of centrifuged and non-centrifuged culture samples were compared and it was found that the cell-free samples consistently had a slightly higher activity (about 10 %) than samples containing cells. The reason for this is not clear, but it shows at least that no active proteinase is bound to the surface of the cells.

The extracellular proteinase of *Serratia* may be purified by gel filtration of culture filtrates. Fig. 2 shows the result of a run on Sephadex G-100, where the enzyme was considerably retarded. For this experiment one liter of cell-free medium from a culture growing in a medium 63 containing 1 % gelatine and 0.1 % peptone was first concentrated about 50-fold by ultracentrifugation before it was applied to the column. Although it is difficult to draw conclusions about the molecular weights of proteins from gel filtration experiments of this kind, it is likely that the proteinase is a relatively small molecule with a molecular weight of about 30 000.

A detailed study of the chemical and catalytic properties of the *Serratia* proteinase has not yet been made. We have tried to purify it by ammonium sulfate fractionation, and chromatography on DEAE-Sephadex according to a similar procedure as that developed earlier for a proteinase from *Arthrobacter*.[9] Most impurities could be removed in this way, but the recoveries were poor and we have not yet been able to crystallize the enzyme. One reason for this is probably that the proteinase is easily autodigested, especially in the absence of Ca^{2+}, which appears to stabilize it.[2,3] The enzyme has a wide catalytic specificity,[3] but we have not been able to show that it hydrolyzes poly amino acids such as poly-L-lysine and poly-L-aspartic acid. Incubation of the partially purified enzyme with various di- and tripeptides did not indicate any hydrolysis.

Prodigiosin pigments are released from *Serratia* towards the end of growth cycles in liquid media. Enzymes which are normally cell-bound can also be detected in the medium of stationary phase cultures. Gel filtration of such culture filtrates have thus given chromatograms in which a low caseinolytic activity and peptidase activities were detected in the void volume peak. An enzyme which cleaves amino acid naphthyl amides was furthermore eluted at about 1.7 void volumes. Also this enzyme appears to be released into the medium as a result of autolysis.

2. Cell bound peptidase. The extracellular proteinase of *Serratia* degrades proteins and polypeptides to oligopeptides. In order to utilize exogenous proteins and large peptides for nutrition, the bacteria must therefore form other enzymes which hydrolyze peptides to free amino acids. Cell extracts were also found to catalyze the hydrolysis of a large variety of peptides. However, when the peptidase activity of cells grown in a complex medium containing gelatine and peptone was compared with the activity of cells grown in medium

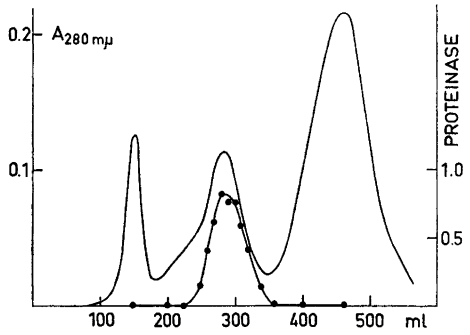

Fig. 2. Gel filtration of a concentrated culture filtrate. 5 ml were applied to a 4 × 48 cm column of Sephadex G-100. The material distribution in the eluate was estimated at 280 mμ (upper curve) and the proteinase activity is shown in the lower curve.

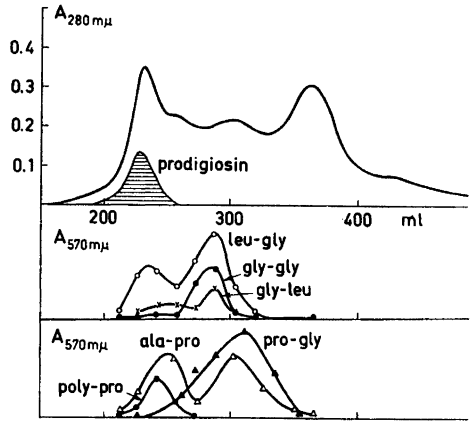

Fig. 3. Gel filtration on Sephadex G-100 of a particle-free cell extract of *Serratia* obtained by sonication and high speed centrifugation. The column had a void volume of about 230 ml and the elution was carried out with 0.1 M Tris-HCl buffer, pH 8, containing 10^{-3} M $MgCl_2$. The peptidase assays were carried out using different incubation times and different peptides, and the heights of the activity curves do not therefore represent true measures of the relative rates at which different peptides were hydrolyzed by the fractions.

63 with glycerol as carbon source there was only a slightly lower activity in the latter type of extracts.

Some fractionation experiments were carried out with extracts obtained by sonication of cells grown on gelatine and peptone. The peptidases in such cell homogenates were not sedimented upon ultracenrtifugation at 105 000 *g* for 60 min, which shows that the enzymes are in true solution after sonication. Peptidases usually require divalent metal ions for stability and activity, but different enzymes bind such ions more or less firmly.[10] Dialysis of *Serratia* extracts against buffers of neutral pH caused only a weak decrease in their peptidase activity, and addition of 10^{-3}—10^{-4} M Mg^{2+} or Mn^{2+} to the assay solution did not increase the rate of hydrolysis of leu-gly. Dialysis against 0.1 M Tris-HCl, pH 8, containing 10^{-3} EDTA resulted in complete disappearance of the activity of the enzymes which cleave leu-gly. However, 10—50 % of the activity could be recovered by incubation of the dialyzed enzyme solution with Mg^{2+} and Mn^{2+}.

Fig. 3 illustrates the great complexity of gel filtration diagrams obtained when cell extracts were fractionated on Sephadex G-100. Earlier experiments [4] have already shown that *Serratia* contains an enzyme which cleaves several amino acid naphthyl amides but not leu-gly and similar simple peptides.

Extracts also contain a high molecular weight component which hydrolyzes leu-NH_2, leu-gly and leu-gly-gly to free leucine and therefore may be classified as a true leucine aminopeptidase. This enzyme elutes with the void volume material in gel filtrations (peak A in Fig. 3). A weak caseinolytic activity could also be detected in this region but this cannot be due to the presence of the peptidase, because these two activities could be separated by chromatography on DEAE-Sephadex.

Peptides containing N-terminal glycine residues (*e.g.* gly-gly, gly-gly-gly, gly-leu and gly-asp) were hydrolyzed very slowly by fractions containing the void volume material. Later fractions (peak B in Fig. 3) cleaved these peptides as well as leu-gly more rapidly.

The hydrolysis of peptide bonds involving proline appear to require specific enzymes depending on whether imino or imido bonds are hydrolyzed.[10] The distribution of activities against ala-pro, pro-gly and poly-pro is also shown in Fig. 3. Our results show that *Serratia* contains both types of enzymes.

Starch gel electrophoresis in glycine-NaOH buffer, pH 8.8, revealed two components active against leu-gly, and both of these also hydrolyzed gly-leu. In addition, a component which cleaves pro-leu was clearly separated from these two enzymes.

The red prodigiosin pigments in *Serratia* could be completely adsorbed by filtration of extracts through DEAE-Sephadex A-50 in equilibrium with 0.1 M Tris-HCl, pH 8. The small amount of caseinolytic enzyme present in the extracts was not adsorbed but could be eluted with the same buffer, whereas no peptidase activity could be detected in the eluate. Stepwise elution with increasing concentrations of NaCl in the buffer resulted in the elution of most, of the peptidase which hydrolyzed leu-gly.

DISCUSSION

Our experiments show that *Serratia* secretes a true extracellular proteinase of relatively low molecular weight. The enzyme is synthesized at a high rate in media containing proteins and peptones, and such compounds have been postulated to act as inducers of the proteinase synthesis.[2] However, as discussed in more detail in another report [11] dealing with the regulation of proteinase formation in *Arthrobacter*, induction of extracellular enzymes has not yet conclusively been shown to occur. The absence of appreciable proteinase formation in media containing easily metabolized compounds such as carbohydrates may be due to catabolic repression of the proteinase formation. It is not known whether this type of unspecific repression acts at the gene level or ribosome level during the enzyme formation.

Proteolytic enzymes found in cell extracts may have other metabolic functions than catalyzing the hydrolysis of exogenous nutrients. A possible anabolic function of peptidases could be in the formation of cell walls, and it has recently been found that specific peptidases play a role in this connection.[12] Intracellular protein turnover may also be a process which is of importance under certain physiological conditions. However, in the case of strongly proteolytic organisms such as *Serratia* it is safe to assume that one or several

different peptidases are of special importance in the catabolism of peptides taken up from the medium.

The ease with wchih *Serratia* can be grown in large quantities may make it a useful source of peptidases, which are of considerable interest to the protein chemist. The leucine aminopeptidase of this organism appears to be more stable than the enzyme purified from animal sources, but further studies on its chemical properties and substrate specificity are obviously necessary.

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REFERENCES

1. Massieu, M. S. *Rev. Latinoam. Microbiol.* **1** (1958) 137.
2. Castañeda-Agullo, M. *J. Gen. Physiol.* **39** (1956) 369.
3. McDonald, I. J. and Chambers, A. K. *Can. J. Microbiol.* **9** (1963) 871.
4. Tjeder, A. and v. Hofsten, B. *Acta Chem. Scand.* **21** (1967) 1721.
5. v. Hofsten, B. and Tjeder, C. *Biochim. Biophys. Acta* **110** (1965) 576.
6. Matheson, A. T. and Tattrie, B. L. *Can. J. Biochem.* **42** (1964) 95.
7. Sarid, S., Berger, A. and Katchalski, E. *J. Biol. Chem.* **234** (1959) 1740.
8. Lewis, W. H. P. and Harris, H. *Nature* **215** (1967) 351.
9. v. Hofsten, B., Van Kley, H. and Eaker, D. *Biochim. Biophys. Acta* **110** (1965) 585.
10. Smith, E. L. and Hill, R. L. In Boyer, P. D., Lardy, H. and Myrbäck, K., (Eds.), *The Enzymes*, Academic, New York 1960.
11. Bjare, U. and v. Hofsten, B.
12. Strominger, J. L., Izaki, K., Matsuhashi, M. and Tipper, D. J. *Federation Proc.* **26** (1967) 9.

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