Characterisation of a Neutral Protease Produced by *Micrococcus luteus*

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A proteolytic enzyme produced by a cassava-fermenting strain of *Micrococcus luteus* was extracted and purified 50-fold by gel filtration and ion exchange chromatography.

The optimum pH for the enzyme was 7.0, the optimum temperature 25 °C, the apparent molecular weight 42 kDa and the K_m value, 0.45 mg ml^{-1} with casein as substrate. The enzyme was stimulated by Ca^{2+} and Mg^{2+} but inhibited by Zn^{2+} and Co^{2+} ions. Other inhibitors were EDTA, KCN, citric acid and L-cysteine indicating the enzyme to be a metalloprotease.

Introduction

Extracellular proteases with a wide variety of specificities are known, but very few are produced industrially. The most important are the alkaline serine proteases and neutral metalloproteases which are formed by *Bacillus* species and the acid proteases from *Aspergillus* and *Mucor* (Aunstrup, 1979). The neutral proteases have received much less research attention as well as commercial applications than the serine proteases. Those from *Bacillus* sp have shown differences in their properties according to the source species (Keay and Wildi, 1970). However, the enzymes have found extensive uses in brewing where their properties match with the conditions used in mashing and are not susceptible to a barley inhibitor which affects serine proteases (Aunstrup, 1980).

The development of industrialised enzyme processes in Nigeria has prompted the search for enzyme-producing bacterial strains with potential applications. This paper therefore presents the properties of a proteolytic enzyme from a food-fermenting strain of *Micrococcus luteus*.

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Materials and Methods

Bacterial strain and culture conditions

Micrococcus luteus strain CSW 304 was isolated from cassava steep liquor as described by Amund and Ogunsina (1987). The organism was grown at 30 °C for 48 h in a mineral salts medium previously described by Amund *et al.* (1990) which contained: KCl, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; $MgSO_4.7H_2O$, 0.5 g; $NaNO_3$, 3.0 g; KH_2PO_4, 1,0 g; $CaCl_2.2H_2O$, 0.1 g; glucose, 10.0 and soluble casein (E. Merck, Germany), 10.0 g in 1.0 litre of distilled water. Cells were harvested from the liquid culture by centrifugation at 10,000 x *g* (4 °C, 25 min) while the supernatant was used as the enzyme source.

Protein and enzyme assays

Protein assay was carried out using the method of Lowry *et al.* (1951) or by optical density measurement at 280 nm. Proteolytic activity was assayed by the method of Keay and Wildi (1970). One millilitre (1.0 ml) of the enzyme was incubated with casein solution (2.0 ml, 2% w/v) at 37 °C for 10 min. The final casein concentration was 13.3 mg ml^{-1}. The reaction was terminated by the addition of 0.4 M trichloroacetic acid (TCA) solution (2.0 ml). The mixture was further incubated at 37°C for another 20 min to fully develop the colour and the absorbance was measured at 660 nm. A blank was prepared by the same procedure, the TCA being added at zero time and the casein after ten minutes of incubation. Absorbance reading was then extrapolated to a tyrosine standard curve. One unit of protease activity was defined as the amount of enzyme that hydrolyses casein with the release of 1.0 nM of tyrosine per minute which is equivalent to an absorbance change of 0.001 at 660 nm under the assay conditions.

Factors affecting enzyme activity

Enzyme assays were carried out at different pH values (3.5–9.0) and different temperatures (20–60 °C). Thermostability of the enzyme was determined by maintaining the enzyme at 50 °C for 120 min while enzyme activity was assayed at regular intervals (10 min). The effect of substrate concentration was determined using different concentrations of casein (0.1–1.5% , w/v) while the effect of

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different cations including Ca^{2+}, Mg^{2+}, Co^{2+} and Zn^{2+}, (0.5–60 mM) on enzyme activity was determined. The effects of inhibitors including EDTA, KCN, citric acid and L-cysteine at different concentrations (2–70 mM) were also examined.

Enzyme purification

Powdered ammonium sulphate was added to 100 ml of culture supernatant to give 80% saturation and the mixture was allowed to precipitate for 2 h at 4 °C . It was then centrifuged for 30 min at 10,000 x *g* and the precipitate was re dissolved in sodium phosphate buffer (0.05 M, pH 7.0) and dialysed against three changes of the buffer at 4 °C for 12 h. A column (1.2 x 40 cm) was packed with Sephadex G-100 and equilibrated with the sodium phosphate buffer. The dialysed precipitate was fractionated on the Sephadex column using the equilibration buffer as described by Andrews (1964). The fractions collected (3 ml) were assayed for their protein contents and enzyme activity. Fractions showing enzyme activity were pooled and rechromatographed on a DEAE-cellulose (Sigma, USA) column using sodium phosphate buffer (0.05 M, pH 7.0) containing an NaCl concentration gradient (0.1–0.4 mM) and the collected fractions assayed for protein content and enzyme activity.

Molecular weight determination

The molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-100 column which was calibrated using the following markers; bovine serum albumin (62 kDa), ovalbumin (45 kDa), chymotrypsinogen (24 kDa) and ribonuclease (13 kDa). All the standard proteins were purchased from Pharamacia Fine Chemicals (Sweden).

Results and Discussion

Production of extracellular protease in culture by *M. luteus* increased exponentially with incubation time reaching a peak during the stationary phase of growth (Fig. 1). Enzyme activity in vitro increased with increasing substrate concentration and reaching a maximum activity at 0.5% (w/v). The K_m and V_{max} values were derived from a typical Lineweaver-Burke plot as 0.45 mg casein

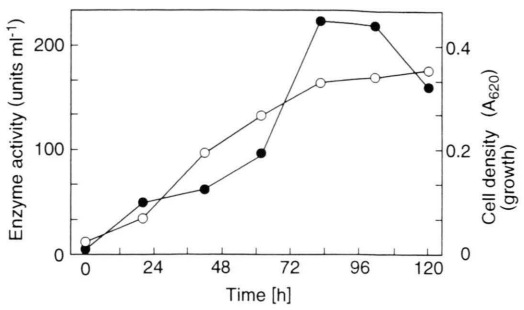

Fig. 1. Time course of protease production by *Micrococcus luteus*. ○, Cell density; ●, Protease activity.

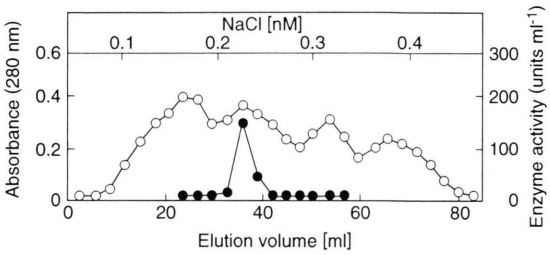

Fig. 2. Ion exchange chromatography of the enzyme fraction obtained from culture filtrates of *M. luteus* on DEAE-Cellulose.
○, Protein concentration;
●, protease activity.

ml^{-1} and 110 units ml^{-1} respectively. The proteolytic enzyme produced by *M. luteus* had properties similar to that of *Lactobacillus brevis* previously isolated and described in our laboratory (Amund *et al.*, 1990) but differs from the serine proteases of *Bacillus* and *Aeromonas* species (Keay and Wildi, 1970; Mellergaard, 1983). Since the majority of *Micrococcus* are soil-borne organisms, it is of physiological importance that they possess proteolytic enzymes to enable them hydrolyse complex protein components present in their natural ecosystems.

A 50-fold purification of the enzyme was achieved as shown in Table I while the enzyme was eluted as a single peak from the DEAE-cellulose column (Fig. 2). The relative purification achieved for protease of *M. luteus* compared favourably with what was earlier reported for *Lactobacillus brevis* (Amund *et al.*, 1990). The optimum pH for the enzyme was 7.0 while the optimum temperature was 25 °C. A 50% inactivation of the enzyme occurred within 10 min of heat treatment at 40 °C whereas at 50 °C, about 95% of enzyme activity

Table I. Purification steps and recovered activities for the proteolytic enzyme produced by *Micrococcus luteus*.

Purification step	Protein [ug ml^{-1}]	Enzyme activity [unit ml^{-1}]	Specific activity [unit ug^{-1} protein]	Relative purification factor
Crude enzyme	255.5	50.0	0.2	1.0
$(NH_4)_2SO_4$ precipitate	102.5	72.0	0.7	3.6
Dialysis	76.0	85.0	1.1	3.7
Sephadex G-100	22.5	145.0	6.4	32.9
DEAE-cellulose	16.5	163.0	9.9	50.4

was lost within 10 min while it was completely denatured after 20 min at the latter temperature. An activation energy value (E_a) calculated from a typical Arrhenius plot was 33.8 kJ mol^{-1}. The molecular weight of the enzyme, estimated by gel filtration to be approximately 42 kDa, was similar to that of the protease of *Psuedomonas aeruginosa* (Inoue *et al.*, 1963) but higher than those of *Lactobacillus brevis* (Amund *et al.*, 1990) and *Aeromonas proteolytica* (Wilkes and Prescott, 1976).

Enzyme activity was enhanced by Ca^{2+} and Mg^{2+} ions up to their optimum concentrations of 0.4 and 1.0 mM respectively. However, Zn^{2+} and Co^{2+} ions were inhibitory at all concentrations employed (0.5–3.0 mM).The enzyme was also inhibited to varying degrees by chemical inhibitors including EDTA, citric acid, KCN and L-cysteine (Fig. 3). Several enzymes are known to be inhibited by EDTA and such enzymes require metallic ions for their activity (Webb, 1963). It is therefore plausible that the proteolytic enzyme of *M. luteus* which is susceptible to EDTA inhibition is a metalloprotease. The amino acid composition and regulation of protease production in *M. luteus* are subjects of further investigation in our laboratory.

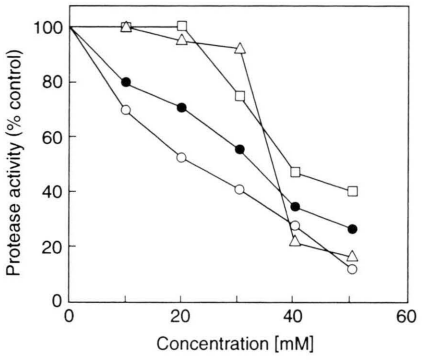

Fig. 3. Effects of chemical inhibitors on the activity of purified protease of *M luteus*.
○, KCN; ●, citric acid; △, EDTA; □, L-cysteine.
The rate for uninhibited enzyme activity (control) was 230 units ml^{-1} (100% activity).

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