Biochimica et Biophysica Acta, 485 (1977) 424—433

BBA 68285

SOME PROPERTIES OF THE EXTRACELLULAR PROTEASE PRODUCED BY THE PSYCHROTROPHIC BACTERIUM *PSEUDOMONAS FLUORESCENS* STRAIN AR-11

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(Received April 22nd, 1977)

Summary

The major extracellular protease from *Pseudomonas fluorescens* strain AR-11 has been partially purified by a factor of 300 by a combination of DEAE-cellulose ion-exchange chromatography and gel filtration. The enzyme had a molecular weight of 38 400 and exhibited optimum activity with isoelectrically precipitated casein substrate at pH 6.5 with K_m = 0.13 mM. The protease was strongly inhibited by a number of heavy metal ions at the 10 mM level and also inhibited by thiol agents, while 10 mM EDTA led to slight activation. Optimum activity was exhibited at an incubation temperature of about 35°C and above 37°C the enzyme was rapidly inactivated, but at low temperatures considerable activity was retained, amounting to 33% of the maximum activity at 4°C and 72% at 20°C. Heat inactivation studies in which the isolated protease was heated at high temperature before subsequent incubation at 35°C with substrate showed that for 50% inactivation 25 s heating at 130°C or 17 s at 140°C or 8.5 s at 150°C was required. The combination of high stability to heat treatments and retention of considerable activity at low incubation temperatures indicates that such a protease might have considerable significance in the processing and subsequent storage of food and other products.

Introduction

Modern methods of milk collection and handling often require the storage of bulk milk in insulated tankers and silos at temperatures of 4—10°C for several days before pasteurisation or other forms of heat treatment. Such conditions

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selectively favour the growth of psychrotrophic bacteria, which can continue to multiply at these temperatures. While subsequent heat treatments may give sterile products, it has recently become clear that many such organisms produce extremely heat stable proteases [1—8] and lipases [8,9] capable of surviving heating processes.

Prominent among the psychrotrophic bacteria producing heat-resistant proteases are the pseudomonads but while the continued action of proteases from these organisms has been implicated in the spoilage of sterile or refrigerated foods and food products comparatively little is known of the properties of these enzymes in biochemical terms. Recently, Adams, Barach and Speck, using either simple ammonium sulphate fractions of broth cultures [1] or unfractionated cultures grown in skimmed milk [2], have examined some of the properties of the proteases produced by ten species of *Pseudomonas* and demonstrated the marked heat stability of these enzymes [1]. Caseins were generally more rapidly hydrolysed than the whey proteins α-lactalbumin and β-lactoglobulin [2] but considerable interspecies differences were observed [1,2]. Preliminary experiments reported from our laboratories [10] using another strain (AR-11) of *Ps. fluorescens* were consistent with the findings of Adams et al. [1,2] and showed that the protease was an extracellular enzyme capable of hydrolysing κ-casein rapidly to *para*-κ-casein, β-casein rather less rapdily and α_{s1}-casein only slowly. The present paper reports an extension of these experiments in terms of the biochemical properties of partially purified preparations of the protease.

Materials and Methods

Preparation and purification of Ps. fluorescens protease

Cultures (500 ml) of *Ps. fluorescens* strain AR-11 were grown with aeration in a nutrient broth containing 1% (w/v) Lab Lemco (Oxoid), 1% (w/v) bacteriological Peptone (Oxoid) and 0.5% (w/v) NaCl for 48 h at 22°C in an orbital incubator rotating at 120 rev./min. The bulk of the bacterial cells were then removed by centrifugation at 2000 × *g* for 30 min and any remaining cells removed from the supernatant by Seitz filtration. Batches of supernatant (500 ml) were concentrated to 30—50 ml by dialysis at 4°C against polyethylene glycol (Carbowax 20 M: Union Carbide U.K. Ltd., Hythe, Hants., U.K.). The concentrate was placed in 9/16-inch d Visking tubing and further dialysed against H_2O overnight at 4°C to reduce the concentration of small molecular weight components of the culture medium, and then chromatographed on a column (20 × 260 mm) of DEAE-cellulose (Whatman DE-52) made up in 0.05 M KH_2PO_4 buffer adjusted to pH 6.5 with 1 M NaOH. After application of the sample, the column was washed with 50 ml of this buffer and enzyme then eluted using a linear gradient of increasing NaCl concentration from 0 to 1 M made up in this same buffer and with 150 ml in each reservoir. Fractions of 5 ml were collected.

Enzyme-containing fractions were pooled, placed in 1/4-inch d Visking tubing, concentrated to 2—3 ml by dialysis against polyethylene glycol and further purified by gel filtration through a column (22 × 550 mm) of Sephadex G-100 using the 0.05 M KH_2PO_4 buffer pH 6.5 for column equilibration

and subsequent elution at a flow rate of approx. 10 ml/h with collection of 3-ml fractions.

Fractions were monitored at 280 nm for protein content and enzyme activities were measured by mixing 0.5 ml of each fraction with 0.5 ml of 0.1 M KH_2PO_4 buffer pH 6.5 and 1.0 ml of casein solution (20 mg/ml in H_2O, the pH being adjusted to 6.5 with 1 M NaOH or 1 M HCl if necessary) containing 0.01 M NaN_3 as preservative. 2 drops of $CHCl_3$ were added to each reaction tube as a further preservative and the tubes were capped and incubated at 35°C for 17 h. Portions (0.5 ml) of 10% trichloroacetic acid solution (w/v) were then added and after standing for 30 min at room temperature the solutions were filtered (Whatman No. 1 paper). Proteolytic activity was determined by measuring the amount of trichloroacetic acid-soluble casein breakdown fragments using a modification of the method of Lowry et al. [11] in which 0.5 ml of trichloroacetic acid filtrate was diluted with 1.0 ml H_2O and mixed with 1.5 ml reagent C [11] and 0.2 ml Folin reagent, before allowing to stand for 30 min and reading absorbance (A) at 750 nm. Casein for use as substrate was isoelectrically precipitated total casein, prepared as described previously [12]. Making the assumption that for many proteins an A_{280} of 1.00 corresponds to concentrations of close to 1.0 mg/ml, specific activities were calculated on the following basis:

$$\text{specific activity} = \frac{D \cdot A \text{ at } 750 \text{ nm}}{t \cdot A \text{ at } 280 \text{ nm}} \text{ units/mg}$$

where D is the dilution factor of the original enzyme solution (32 in the above) and t is the incubation time in hours.

Analytical procedures

In addition to being used preparatively as described above, the Sephadex G-100 column was also used for molecular weight estimation [13], for which the column was standardised with the following proteins of known molecular weight: α-lactalbumin, chymotrypsinogen, ovalbumin and bovine serum albumin. Conditions were the same as those used for the protease purifications.

For measurements of the pH optimum 0.2-ml samples of enzyme solution were mixed with 1.0 ml of casein substrate (30 mg/ml in H_2O) and 0.8 ml of buffer and incubated for 17 h at 37°C. Buffers were 0.25 M potassium hydrogen phthalate/0.25 M NaOH over the pH range 4.8—5.8, 0.25 M potassium dihydrogen phosphate/0.25 M NaOH from pH 6.0 to 7.6 and 0.25 M Tris · HCl from pH 7.6 to 8.2. Two drops $CHCl_3$ were added to all tubes as a preservative. The amount of trichloroacetic acid-soluble material after the incubation was determined as above.

The enzyme activity vs. incubation temperature profile was measured in a similar manner in which 0.3-ml samples of protease solution were mixed with 1.0 ml of casein substrate (20 mg/ml in H_2O containing 0.01 M NaN_3), 0.7 ml of 0.25 M KH_2PO_4 buffer adjusted to pH 6.3 with 1 M NaOH and 2 drops $CHCl_3$. Incubation for 17 h at appropriate temperatures in a series of water and constant-temperature rooms was followed by measurement of trichloroacetic acid-soluble material as above.

For heat-inactivation studies 70—80 μl portions of protease solution were drawn up into 100 μl Drummond Microcap disposable glass pipettes (Shandon

Southern Instruments Ltd.) and both ends of the tubes flame-sealed. This capillary tubing was of sufficiently uniform internal diameter for solution volumes to be taken as proportional to length. For each experiment a series of such tubes was placed in a stirred oil-bath at the desired temperature, tubes were withdrawn after suitable heating times and immediately plunged into cold water. The length of the liquid column in each cooled tube was measured and the tubes were then crushed with a glass rod into individual test-tubes containing 1.0 ml of 0.25 M KH_2PO_4 buffer pH 6.5. Casein substrate (1.0 ml, 20 mg/ml in H_2O) containing 0.01 M NaN_3 was added to each tube, together with 2 drops $CHCl_3$, and incubation at 35°C was carried out for 17 h prior to estimation of trichloroacetic acid-soluble material as above. The amounts of proteolytic activity found (absorbances at 750 nm) were divided by the length of liquid in the cooled capillary tube before crushing to give the activity/cm which was then expressed as a percentage of the activity/cm of controls carried through the above procedure but omitting the heating step.

For the inhibition experiments 0.2 ml of the protease solution was mixed with 0.5 ml of 0.2 M Tris/maleate buffer pH 6.5 and 0.7 ml of inhibitor solution (0.03 in H_2O). After 30 min pre-incubation at 37°C, 0.7 ml of casein substrate solution (30 mg/ml) in the Tris/maleate buffer pH 6.5 was added, together with 2—3 drops of $CHCl_3$, and incubation continued for a further 17 h at 37°C. Trichloroacetic acid-soluble material was then estimated as above. The chymotrypsin inhibitor 2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl carbamate and the trypsin inhibitors from soybean, egg-white (ovomucoid) and beef pancrease were all obtained from Sigma Chemical Co. Ltd.

Results

The separation of the protease of *Ps. fluorescens* AR-11 achieved by ion-exchange chromatography on the DEAE-cellulose column is shown in Fig. 1. Small amounts of activity either did not adhere to the column or were eluted at very low ionic strengths and may represent either minor proteolytic components or the enzyme in a bound form. The major peak was eluted at an ionic strength of about 0.20—0.25 M in the NaCl gradient and was well separated from the bulk of 280 nm-absorbing material and from much of the coloured material present in the culture extract. Protease-containing fractions were pooled as indicated (Fig. 1) and further purified by gel filtration. This led to a further separation of the enzyme from inactive protein (Fig. 2) and also removed any residual traces of coloured material. Active fractions were pooled as indicated (Fig. 2). Table I shows the effectiveness of this partical purification scheme in terms of yield and specific activities as defined in Materials and Methods. Overall recovery was about 50%. Although this enzyme material was used routinely without further purification, for some experiments (such as the heat-inactivation studies) enzyme with a higher specific activity was helpful and for this a second cycling through the Sephadex G-100 gel filtration column was used, the conditions being unchanged from the first cycle. This resulted in a pooled peak of enzyme activity with a specific activity of 28.9 units/mg, and an overall recovery of about 37% with about a 300-fold purification for the best fractions. Clearly, however, this preparative scheme should only be regarded as a partial

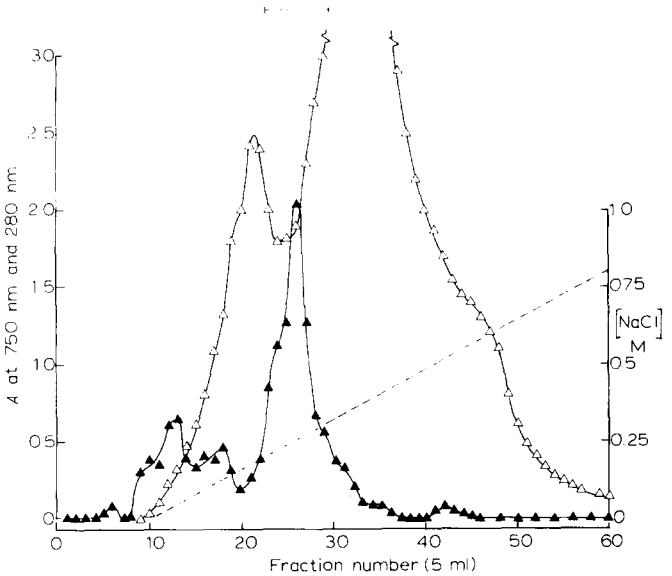

Fig. 1. Ion-exchange separation on a DEAE-cellulose column (20 X 260 nm) of *Ps. fluorescens* AR-11 protease with a linear 0—1 M NaCl elution gradient in 0.05 M KH_2PO_4 buffer pH 6.5. △———△, protein absorbance at 280 nm; ▲———▲, protease activity, absorbance at 750 nm (see text). Enzyme-containing fractions (23—29) were pooled as indicated by the bar.

purification, giving a product sufficiently pure for the purposes of these studies.

The molecular weight of the protease as determined by gel filtration was 38 400 ± approx. 2000, the symmetry of the single peak of the enzyme activity suggested the presence of only one protease component. Very similar results

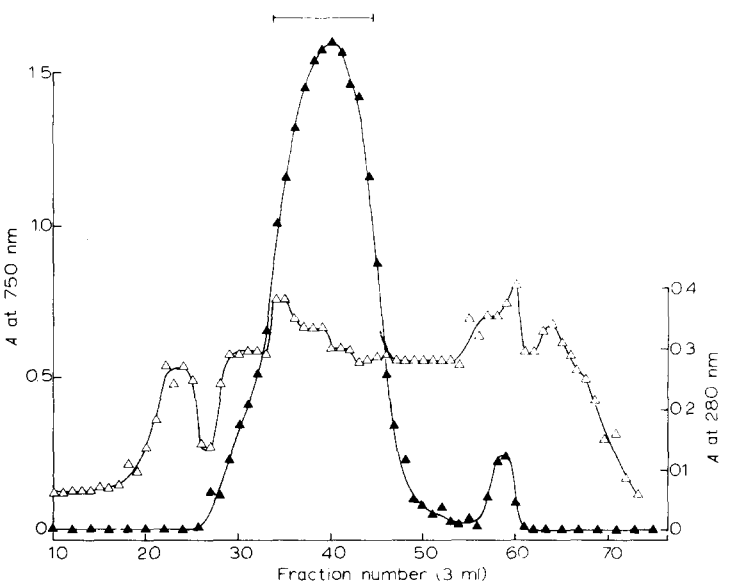

Fig. 2. Gel filtration of AR-11 protease on a column (22 X 550 nm) of Sephadex G-100 in 0.05 M KH_2PO_4 buffer pH 6.5. △———△, protein absorbance at 280 nm; ▲———▲, protease activity, absorbance at 750 nm (see text). Enzyme-containing fractions (34—44) were pooled as indicated by the bar.

TABLE I

PREPARATIVE SCHEME FOR THE PARTIAL PURIFICATION OF *PS. FLUORESCENS* AR-11 PROTEASE

	Proteolytic activity (units *)	Specific activity (units/mg *)
Culture supernatant	37.5	0.1
DEAE-cellulose eluate	27.2	1.5
Gel filtration (1st cycle)	19.1	8.8
Gel filtration (2nd cycle)	14.0	28.9

* See text for definition.

were also obtained in 0.2 M sodium acetate buffer pH 4.5 and in 0.2 M Tris · HCl buffer pH 7.6.

The activity vs. pH curve (Fig. 3) showed that optimum activity was at pH 6.5. Although the curve is the mean of three experiments it is not symmetrical and shows apparent shoulders on both sides of the pH optimum, which may suggest some enzyme heterogeneity although the deviations from symmetry are almost within the experimental error of the assay procedure and may not be significant. Possible effects of pH on the geometry or state of aggregation of the casein substrate may also explain the lack of symmetry (casein will exist in an aggregated form in such aqueous buffers with apparent molecular weights of the order of 10^6 or more).

The curve of proteolytic activity vs. temperature of incubation is shown in Fig. 4 from which it can be seen that the optimum was about 35—36°C. Above 37°C the enzyme rapidly lost activity and at 45°C only exhibited about 25% of its maximum activity. This was perhaps due at least in part to inactivation by denaturation during the comparatively long incubation period. At lower temperatures however the enzyme retained considerable activity, so that at a typical refrigeration temperature of 4°C it possessed about 33% of the maximum.

The results of the heat inactivation studies involving heating at a high temperature for a short time followed by measurement of residual protease activity

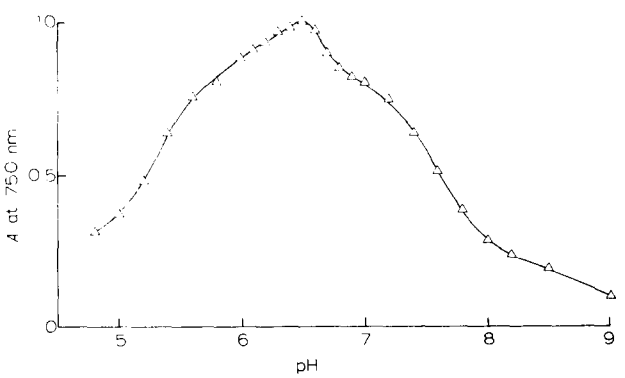

Fig. 3. Protease activity vs. pH curve using isoelectrically-precipitated casein substrate (15 mg/ml) in 0.25 M buffers (see text for details).

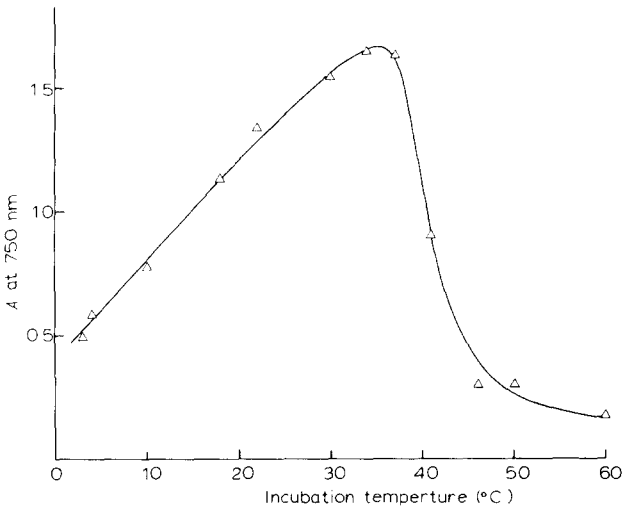

Fig. 4. The influence of incubation temperature on the hydrolysis of isoelectrically-precipitated casein (10 mg/ml) by AR-11 protease in 0.09 M KH_2PO_4 buffer pH 6.5.

at 35°C are shown in Fig. 5 from which the marked heat-stability of the enzyme can be seen. An Arrhenius plot of log $(1/t)$ vs. $1/T$, where t is the time required for inactivation at temperature T (in K) based on the data in Fig. 5, gave a linear relationship, the slope corresponding to an activation energy (E) for the denaturation reaction of $9.28 \cdot 10^4$ J · mol^{-1}. It was possible however for the data to be interpreted within experimental error as non-linear, with two linear segments of slopes corresponding to $E = 1.33 \cdot 10^5$ J · mol^{-1} at the higher

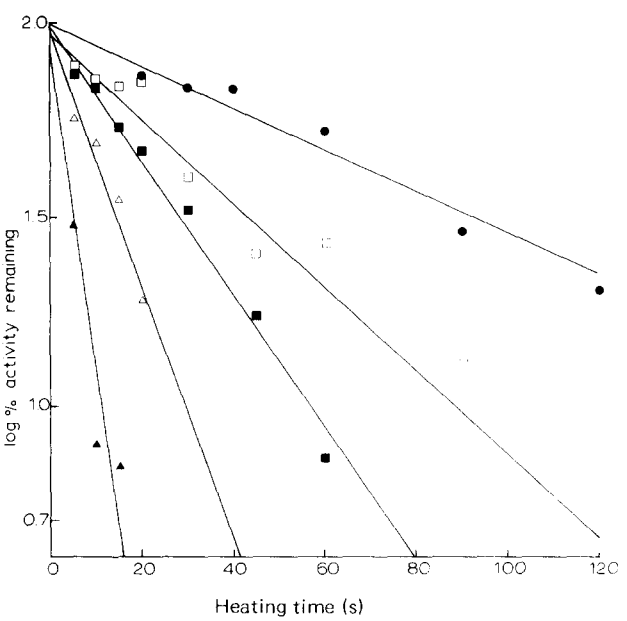

Fig. 5. The heat-inactivation of AR-11 protease in 0.05 M KH_2PO_4 buffer pH 6.5 at temperatures of: ●——●, 120°C; □——□, 130°C; ■——■, 140°C; △——△, 150°C and ▲——▲, 160°C.

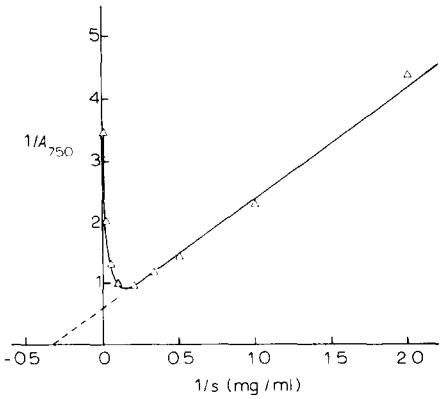

Fig. 6. Lineweaver-Burk plot for the hydrolysis of isoelectrically-precipitated casein by AR-11 protease in 0.05 M KH_2PO_4 buffer pH 6.5. The broken line is the extrapolation of the linear portion of the plot to give the intercept on the $1/s$ axis corresponding to $-1/K_m$.

temperatures (usually the homogeneous mode of a non-linear reaction) and $E = 7.82 \cdot 10^4$ J · mol^{-1} at the lower temperatures.

Inhibition experiments showed that under the conditions employed AR-11 protease was almost completely inhibited by a number of metal ions, such as Ag^+, Al^{3+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+}, and less strongly (15—60%) inhibited by the reagents reacting with SH groups; dithiothreitol, iodoacetamide, iodoacetic acid, 2-mercaptoethanol, *N*-ethylmaleimide and *p*-chloromercuribenzoate. Ethylenediaminetetraacetic acid (EDTA) slightly enhanced activity, probably by removal of traces of inhibiting metal ions. The protease was not inhibited by F^- or by the trypsin and chymotrypsin inhibitors.

With isoelectrically precipitated casein as substrate in 0.05 M KH_2PO_4 buffer pH 6.5 the rate of substrate hydrolysis by the protease was examined over a concentration range of 0.25—100 mg/ml. This was found to be proportional to concentration below 5.0 mg/ml and a linear Lineweaver-Burk plot of $\frac{1}{v}$ vs. $\frac{1}{s}$ was obtained (Fig. 6), where v is the velocity of the reaction and s the substrate concentration. The least mean squares plot extrapolating the linear portion of the curve to $\frac{1}{v} = 0$ gave a K_m value of 3.08 mg/ml, corresponding to about 0.13 mM if the molecular weight of isoelectrically precipitated casein is taken as 23 000. At concentrations greater than 5 mg/ml however there was marked substrate inhibition reflecting interactions between enzyme and substrate at positions removed from the active site. A possible alternative interpretation that there was a fall in substrate availability at high concentrations due to increase in the aggregate size (casein exists in an aggregated form in such aqueous buffers) cannot be ruled out at this juncture.

Discussion

Limited biochemical studies have recently been reported on the proteases from a strain (R-12) of *Ps. fluorescens* isolated from river water [14] and on a strain (MC-60) isolated from raw skim milk [1], but in both cases purification was less extensive than we obtained and our overall recoveries of activity were improved by omission of the rather inefficient ammonium sulphate precipita-

tion step as used by other workers. Juan and Cazzulo [14] reported that the R-12 strain required the addition of Ca^{2+} to the culture medium for production of protease. Starting with a broth which we calculate would have contained about 8 mg/l of Ca^{2+} they added a further 108 mg/l of Ca^{2+} (as 0.3 g/l $CaCl_2$). Although our culture medium contained initially a rather higher level (29 mg/l) of Ca^{2+}, addition of a further 108 mg/l (as 0.3 g/l $CaCl_2$) was without effect on the production of protease by *Ps. fluorescens* AR-11. It is not clear at this juncture whether this represents a genuine difference in requirements between the strains or whether it merely reflects the different Ca^{2+} contents of the initial media.

It is clear from the separation procedure that a single extracellular enzyme constitutes the bulk of AR-11 protease activity. Although small amounts of activity were eluted at low ionic strengths from the DEAE-cellulose column (Fig. 1) before the main peak, the proportion was rather variable and often less than 10% of the total activity. Although not investigated further, this probably suggests that the minor component is the same enzyme as the major peak but in an associated form or bound to some cellular fragment. The molecular weight of the major peak enzyme (38 400) determined by gel filtration was very similar to that of about 37 000 reported [14] for the enzyme from *Ps. fluorescens* strain R-12.

The AR-11 protease possessed wide specificity and in preliminary experiments it was found that haemoglobin and bovine serum albumin were also hydrolysed, but not so rapidly as β-casein or isoelectrically precipitated whole casein. With whole casein we found a comparatively sharp pH optimum at 6.5, falling to about 30% of the maximum at pH 5 or 8 and to about 10% at pH 9, which is in contrast to the very broad optimum over the pH range 6.5—10.0 reported for the R-12 strain [14] and the sharper but rather higher value of 7.5 for *Ps. fluorescens* MC-60 protease [1].

The R-12 protease was reported [14] to be unaffected by 1 mM EDTA or by 2 mM *p*-chloromercuribenzoate, although prolonged dialysis against 5 mM EDTA caused a loss of enzyme activity which was largely regained by addition of Zn^{2+} or Co^{2+}. At the 5-mM level these ions were less effective in reactivating the apoenzyme than at the 1-mM level. Thus our findings that the AR-11 enzyme was inactivated by Zn^{2+} and Co^{2+} and a number of other heavy metal ions may not be inconsistent with their results since we studied only the effect at a concentration of 10 mM. Similar considerations may also explain why we found *p*-chloromercuribenzoate to be inhibitory while they did not, although it seems unlikely that the difference between the effects of EDTA treatment on the two proteases can be due merely to the concentrations used.

The kinetics of the hydrolysis of casein by AR-11 protease showed that since substrate inhibition occurred at casein concentrations above about 5 mg/ml, in bovine milk which contains approx. 25 mg/ml ($1/s$ = 0.04 in Fig. 6) considerable inhibition must be occurring. This means that in this region changes in the concentrations of milk proteins will not greatly alter the rate of casein breakdown in absolute terms (i.e. weight vs. time) and also that in diluted milk systems where little or no substrate inhibition is occurring the relative rate of loss of casein to that remaining unhydrolysed will be greater than that seen in the initial milk or in more concentrated products.

The effect of temperature of incubation on the hydrolysis of casein was very different for the AR-11 protease by comparison with the MC 60 enzyme. Adams et al. [1] reported optimum activity at 45°C for MC-60 protease and at 25°C (the lowest temperature studied) the activity had fallen to about 25% of the maximum. By contrast AR-11 protease (see Fig. 4) had a lower optimum, at 35°C, was rapidly inactivated above this and had much greater activity at low temperatures. The considerable retention of activity at typical refrigeration and storage temperatures suggests that such proteases may be of considerable importance in the keeping qualities of food and other products, the more so since the heat inactivation experiments (Fig. 5) showed that the AR-11 protease would survive any typical pasteurisation conditons and even most sterilisation procedures.

Acknowledgements

We should like to thank Dr. M.E. Sharpe for the initial provision of cultures of *Ps. fluorescens* AR-11 and Dr. B.A. Law for the growth of batches of culture and provision of culture supernatant filtrates. We are indebted to Mr. M.D. Taylor for skilled technical assistance.

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