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Separation and Characterization of Three Proline Peptidases from a Strain of *Arthrobacter*

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Three different enzymes capable of hydrolyzing proline-containing dipeptides were separated by gradient chromatography or by zone electrophoresis. Starch gel electrophoresis also showed different electrophoretic mobilities.

None of the enzymes are activated by the addition of metal ions. Treatment with EDTA completely inactivates two of the three peptidases. The inactivated enzymes can be partly reactivated by addition of Mn^{2+} or Mg^{2+} in one case and Mn^{2+} or Co^{2+} in the other. DFP does not inhibit any of the enzymes.

The peptidases have pH optimum between pH = 7.5 and 8.5 and are stable from pH = 6 to pH = 10.

The enzymes show different substrate specificity, but hydrolyze only dipeptides with few exceptions. Peptides with blocked N-terminals are split very slowly or not attacked at all.

Fairly high activities of the three enzymes are also found after cultivating the bacteria on substrates containing no protein.

Separation of peptidase activities in various biological materials has shown that a variety of enzymes exists, hydrolyzing various peptide bonds. Compared to the animal peptidases, little effort has apparently been directed toward the isolation and study of the peptidases of microbial origin. Most of these microbial enzymes are incapable of attacking bonds on either side of proline.

However, Sarid *et al.*[1-2] isolated a proline iminopeptidase from a strain of *E. coli* K 12 that can cleave off N-terminal proline residues regardless of the length of the peptide chain.

Yaron and Mlynar[3] have described an aminopeptidase (called aminopeptidase – P) releasing only those N-terminal amino acids that are followed by proline residues. Both high- and low-molecular weight peptides are attacked.

The strain of *Arthrobacter* isolated in this Laboratory[4-6] was strongly proteolytic when grown on a medium containing gelatin. Previous work on the extracellular and cell-bound proteases revealed high activity toward several dipeptides. The present report describes the separation, partial purification and some properties of three enzymes capable of hydrolyzing peptides with proline as N- or C-terminal amino acid.

MATERIAL AND METHODS

Organism and culture conditions. The strain of *Arthrobacter* described earlier[4–6] was used. Cultures of bacteria were grown in batches of 16 l. The medium, called M63, contained per liter: KH_2PO_4 (13.6 g), $(NH_4)_2SO_4$ (2.0 g), $FeSO_4.7H_2O$ (0.5 mg), $MgSO_4.7H_2O$ (0.2 g), and $CaCl_2$ (1 mg). The pH was adjusted to 7.4 with KOH.Gelatin(Oxoid Division of Oxo Ltd., London, England) and bactocasitone (DIFCO Laboratories, Detroit, Mich.) were added to final concentrations of 1 % and 0.1 %, respectively. The culture was incubated in a water bath at 30°C, stirred and vigorously aerated with sterile air. Foaming was controlled by addition of Antifoam A (Kebo AB, Stockholm, Sweden).

Preparation of cell free extract. After 24 h the cells were harvested and washed with 0.1 M Tris – HCl – 0.005 M $MgSO_4$, pH 8.0. The washed cells were suspended in the same buffer to a final volume of about 350 ml. Portions of 40 ml were disintegrated for 20 min in a Raytheon 250 W, 10 kc Sonic Oscillator at 5 – 10°C. Cell debris was removed by centrifugation at 105 000 *g* for 60 min in a Spinco Ultracentrifuge. The supernatant fluid contained the crude enzyme preparation.

Protein determination. The method of Folin-Ciocalteu was used as described by Lowry *et al.*[7] Serum albumin (B. D. H. Ltd., Laboratory Chemicals Division, Poole, England) was used as a standard.

Activity assay. The hydrolysis of peptides was followed by the ninhydrin method of Matheson and Tattrie.[8] The release of free proline from proline-containing peptides was measured according to Sarid *et al.*[1] Assays were done at 37°C using 0.8 ml 0.1 M Tris – HCl, pH 8.0, and 100 µl peptide solution (0.5 %). After temperature equilibration, 100 µl enzyme solution was added and aliquots were taken at zero time, and usually after 20 min. All activity assays were conducted at enzyme concentrations and time intervals that gave linear kinetics as determined by pilot experiments. Peptides were obtained from Miles-Laboratories, Inc., Elkhart, Indiana, and Sigma Chemical Co., St. Louis. Some longer peptides were kindly donated by Dr. A. Nordwig, Max Plank Institute, Munich, West Germany.

Ion exchange chromatography. A column (40 × 2.0 cm) packed with DEAE-cellulose (Whatman Microgranular DE-32, W & R Balston Ltd., England) and equilibrated with 0.05 M NaAc – 0.028 M HAc, pH = 5.0, was used. The sample, followed by two total volumes of starting buffer, was applied to the column, which was then developed with a linear gradient of 350 ml 0.05 M NaAc – 0.028 M HAc (mixing chamber) *versus* 350 ml 0.5 M NaAc – 0.28 M HAc (reservoir) at a constant pH of 5.0. The chromatography was carried out at 4°C with a flow rate of 40 – 50 ml/h. Fractions of about 5.5 ml were collected.

Gel filtration. Gel filtration was carried out at 4°C using Sephadex G-200 (Pharmacia, Uppsala, Sweden). The column (95 × 3.2 cm) was equilibrated with 0.1 M Tris – HCl – 0.005 M $MgSO_4$, pH = 8.0. The flow rate was 1.5 – 2.0 cm/h. Fractions of 5 ml were collected.

Zone electrophoresis. Zone electrophoresis was performed on a vertical column (97 × 4 cm) packed with pyridine-washed cellulose powder (Munktell No. 410, Grycksbo Pappers AB, Grycksbo, Sweden) according to the technique described by Porath.[9] The column was equilibrated with 0.075 M Tris-citrate buffer, pH = 8.8. The electrophoresis was run at 5.2 V/cm for 48 h. The column was eluted at a flow rate of 15 – 25 ml/h. Fractions of 2.0 – 2.5 ml were collected.

Starch gel electrophoresis. Starch gel, purchased from Connaught Medical Research Laboratories, Toronto, Canada, was prepared for use as described by Smithies.[10] The electrophoresis was carried out in a horizontal tray in 0.035 M glycine – NaOH buffer, pH = 8.8. The electrode vessels contained 0.35 M glycine-NaOH buffer, pH = 8.8. Samples of 20 µl were applied to filter papers (Whatman No. 3 MM) which were then inserted into slits in the gel. The gel was covered with a sheet of parafilm. The electrophoresis was run for 16 – 18 h at 3.2 V/cm, after which the gel was sliced in the usual manner. Peptidase activities were detected by the method described by Lewis and Harris.[11]

Qualitative analysis of peptide - hydrolysates

(*a*) *Thin layer chromatography.* Conditions for incubation were the same as described under Activity assay. Analytical separations of amino-acids and peptides were performed by TLC. 0.25 mm layers of Silica Gel G (E. Merck AG, Darmstadt, West Germany) were used. The solvent systems propanol:water, 70:30 (v/v) and butanol:acetic acid: water, 4:1:1 (v/v/v) were used. Amino-acids and peptides were detected by spraying with 0.3 % ninhydrin in butanol, containing 3 ml acetic acid per 100 ml. Proline was specifically located by spraying with a solution containing 1 % ninhydrin and 17 % trichloroacetic acid in ethanol.[12]

b) *High voltage paper electrophoresis.* Sometimes, high voltage paper electrophoresis gave better separations than TLC. The equipment used was manufactured by AB Analysteknik, Vallentuna, Sweden. The buffer used was 4 % formic acid, pH = 1.85. The electrophoresis was run for 1½ h at a voltage gradient of 60 V/cm. The spots were detected as described above.

RESULTS

I. Separation of different activities and partial purification

A. *Precipitation at pH = 5.0.* The centrifuged cell extract was dialyzed overnight against 0.05 M NaAc – 0.028 M NaAc buffer, pH = 5.0. The precipitate formed, which represented about 70 – 80 % of the total protein, was centrifuged down and discarded. Although most of the activity toward Pro-Gly remained in the clear supernatant, much of the enzyme activity toward Ala-Pro was lost (see Table 1).

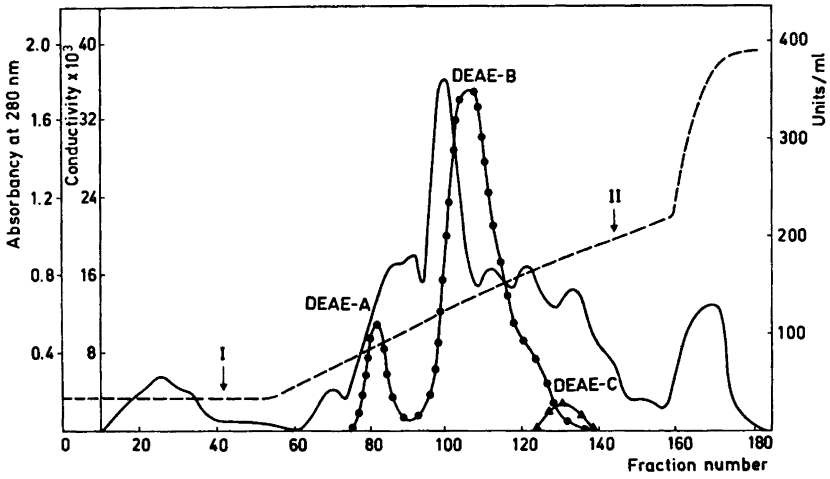

Fig. 1. Ion exchange chromatography on DEAE-cellulose of cell extract, dialyzed against 0.05 M NaAc – 0.028 M HAc buffer, pH = 5.0, and centrifuged. The column (40 × 2.0 cm) was developed by an NaAc-HAc gradient at a constant pH = 5.0, starting at I. At II, a final buffer of 1.0 M NaAc – 0.56 M HAc, pH = 5.0, was applied. (———), absorbancy at 280 nm. (- - -), conductivity. (· — · — ·), activity toward Pro-Gly. (▲), activity toward Ala-Pro. Fractions of 5.5 ml were collected.

B. *Ion exchange chromatography.* The solution was placed on a DEAE-column (see Methods) and eluted with a linear salt gradient (Fig. 1). Two components with activity toward Pro-Gly and one with activity toward Ala-Pro were found. They are called DEAE-A, DEAE-B, and DEAE-C, respectively. DEAE-A splits Pro-Gly, while DEAE-B hydrolyzes a variety of dipeptides, such as Leu-Gly, Gly-Leu, Gly-Gly, as well as Pro-Gly. DEAE-C splits Ala-Pro, and shows slight activity toward Ley-Gly and Pro-Gly, probably owing to contamination with DEAE-B, as the two peaks are not completely resolved. Attempts to fractionate DEAE-B further on DEAE-cellulose with different gradients of salt concentration and pH were unsuccessful. Neither ion exchange chromatography on CM-cellulose (CM-32, Microgranular, W & R, Balston Ltd., England) nor adsorption chromatography on hydroxylapatite gave fractionation of this material. The yield of DEAE-C was always very poor after chromatography on DEAE-cellulose. Apparently, part of the enzyme became denatured and was adsorbed irreversibly to the column.

C. Gel filtration. DEAE-B could be further purified (about 10-fold) by gel filtration on Sephadex G 200 (see Methods). The enzyme elutes at about 1.6 void volumes of the column. If crude cell extract was placed on the same column, the three peptidases all eluted at 1.6 void volumes, indicating similar molecular weights.

D. Zone electrophoresis. Zone electrophoresis of the crude cell extract on cellulose is shown in Fig. 2. Three activity peaks, designated CE-A, CE-B, and CE-C, are evident. CE-A cleaved Ala-Pro and showed no activity toward Pro-Gly and Leu-Gly, provided that only the central fractions of the peak were pooled. The yield was also high compared to the yield of DEAE-C (see Table 2).

E. *Starch gel electrophoresis of the partially purified preparation.* DEAE-A and CE-C have the same electrophoretic mobility in starch gel and are presumed to represent the same enzyme, which will be designated peptidase A. DEAE-B and CE-B also migrated together in the gel and are termed peptidase B. DEAE-C and CE-A are also electrophoretically identical and are called peptidase C. Peptidases A and B could also be shown to be non-identical using starch-gel electrophoresis, where the two peptidases showed different mobilities. Leu-Gly was split only with peptidase B. This activity was found at exactly the same place as the activity resulting from hydrolysis of Pro-Leu by the same enzyme. Peptidase C showed no activity either to Pro-Leu or to Leu-Gly.

Table 3. Effects of various metal ions on the peptidase activity. The activities are expressed as percentage of the controls not treated with EDTA or metal ions. The final metal ion concentration was 10^{-1} M.

Metal	Peptidase A		Peptidase B		Peptidase C	
	Untreated	+ EDTA	Untreated	+ EDTA	Untreated	+ EDTA
Control	100	86	100	3	100	0
Mn^{2+}	85	64	97	24	6	15
Mg^{2+}	101	66	80	24	94	0
Co^{2+}	91	59	36	8	33	33
Zn^{2+}	8	30	75	6	0	0

Table 1. Summary of the separation of the proline-peptidases. Procedure including DEAE-chromatography.

Purification step		Volume (ml)	Total proteins (mg)	Total activity (units)		Specific activity (units/mg)		Recovery (%)		Purification	
1. Cell extract		96	950	Pro—Gly		113 300	Pro—Gly	119	Pro—Gly	100	Pro—Gly	1
				[a] Peptidase A		6 800	A	7.0				
				[a] Peptidase B		106 500	B	112				
				Ala—Pro		21 600	Ala—Pro	22.7	Ala—Pro	100	Ala—Pro	1
2. Supernatant after precipitation at pH = 5.0		81	194	Pro—Gly		110 600	Pro—Gly	569	Pro—Gly	98	Pro—Gly	4.8
				Ala—Pro		6 400	Ala—Pro	32.9	Ala—Pro	30	Ala—Pro	1.5
3. Chromatography on DEAE-cellulose	Peptidase A Pro—Gly	55	7.6			5 500		717		81		102
	Peptidase B Pro—Gly	140	24.5			77 000		3 140		72		28.0
	Peptidase C Ala—Pro	59	8.9			650		73.3		3		3.2

[a] Relative amounts of peptidases A and B were calculated from EDTA-inhibition of activity towards Pro-Gly in crude cell-extract (8 % residual activity) and the two purified peptidases (Table 3).

Table 2. Summary of the separation of the proline-peptidase. Procedure including zone electrophoresis on cellulose.

Purification step		Volume (ml)	Total proteins (mg)	Total activity (units)		Specific activity (units/mg)		Recovery (%)		Purification	
1. Cell extract dialyzed against starting buffer		26.4	81.8	Pro–Gly	36 300	Pro–Gly	450	Pro–Gly	100	Pro–Gly	1
				[a] Peptidase A	2 200	A	27				
				[a] Peptidase B..	34 100	B	423				
				Ala–Pro	9 240	Ala–Pro	113	Ala–Pro	100	Ala–Pro	1
2. Pooled preparations from electrophoresis on cellulose	Peptidase A Pro–Gly	12.3	3.8		800		210		36		7.8
	Peptidase B Pro–Gly	18.0	6.2		25 300		5 770		74		13.6
	Peptidase C Ala–Pro	9.6	3.0		3 140		1 550		34		13.7

[a] Relative amounts of peptidases A and B were calculated from EDTA-inhibition of activity towards Pro-Gly in crude cell extract (8 % residual activity) and the two purified peptidases (Table 3).

II. Properties of the partially purified proline-peptidases

A. *Effects of metal ions on activity.* Pooled preparations from DEAE-chromatography or CM-cellulose electrophoresis were dialyzed against 0.02 M EDTA, pH = 8.0, for 24 h. The EDTA was removed by dialysis against deionized water for 12 h. Part of the solutions was incubated with different metal ions at 37°C for 1 h, and part was used as a control. None of the ions added increased the peptidase activities above the level of the controls (Table 3). Of the ions tested, Mg^{2+} and Mn^{2+} were most effective in restoring the activities of peptidase A and B, while peptidase C was best reactivated by Co^{2+}.

Metal activation was also carried out at 37°C for 1 h without prior treatment with EDTA. None of the metal ions increased the activity over the controls for peptidase A, B, or C. Instead, in almost every case, the values were more or less lower, indicating an inhibitory effect of these ions when present in excess (Table 3).

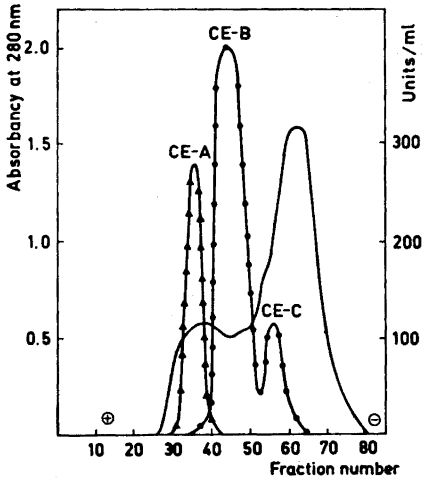

Fig. 2. Zone electrophoresis of concentrated cell extract on a 97 × 4.0 cm column of cellulose in 0.075 M Tris-citrate buffer, pH = 8.8, at 5.2 V/cm for 48 h. (———), absorbancy at 280 nm. (●), activity toward Pro – Gly. (▲), activity toward Ala – Pro. Fractions of 2.0 ml were collected.

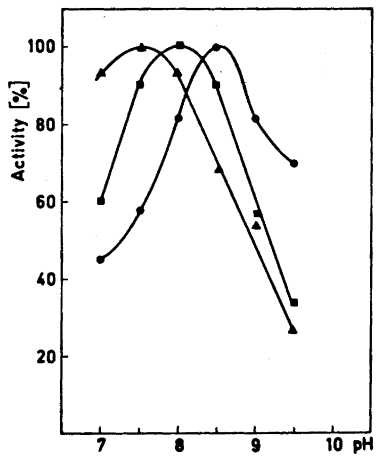

Fig. 3. pH dependences of activity of the three peptidases. (■), peptidase A (substrate Pro – Gly). (●), peptidase B (substrate Pro – Gly). (▲), peptidase C (substrate Ala – Pro). The enzyme solutions were incubated at various pH's for 30 min, after which activity was measured.

B. *Effect of inhibitors.* Neither peptidases A and B, nor C were inhibited upon incubation with diisopropylphosphofluoride (DFP, final concentration 5×10^{-3} M) for 1 h, indicating that none of these peptidases contains serine residues at the active sites.

Partly purified peptidases were also incubated (37°C, 1 h) with *p*-chloromercuribenzoic acid (PCMB, L. Light & Co. Ltd., Colnbrook, England). The final concentration of PCMB was 3×10^{-4} M. Peptidases A and B were not inhibited by this treatment, but peptidase C was completely inactivated.

C. *pH Optimum and stability.* The pH optimum was determined for the three peptidases. 0.1 M Tris-HCl buffers were used from pH = 7 to pH = 9.5. The results are shown in Fig. 3. Peptidase B has its optimum at pH = 8.5, peptidase A at pH = 8, and peptidase C at pH = 7.5.

The peptidases were also dialyzed against buffers with pH-values from pH = 5.0 to pH = 11.0. The activity of the solutions were measured after different time intervals up to one week. 0.1 M citrate-phosphate buffer was used from pH = 5.0 to pH = 7.0, 0.1 M Tris-HCl buffer from pH = 7.5 to pH = 9.0, and 0.1 M glycine-NaOH buffer from pH = 9.5 to pH = 11.0. The three peptidases were stable between pH = 6 and pH = 10, where small changes in activity were observed.

D. *Thermostability.* Solutions of peptidase preparations in 0.1 M Tris-HCl buffer, pH = 8.0, were incubated at 40 or 50°C for 3 h. Samples were taken at several time intervals, and the activity was measured. The results are shown in Fig. 4. Peptidase B is the most heat-stable of the three enzymes with about 35 % loss of activity after 3 h at 50°C. Both peptidases A and C are very labile at this temperature and lost all activity within 30 min.

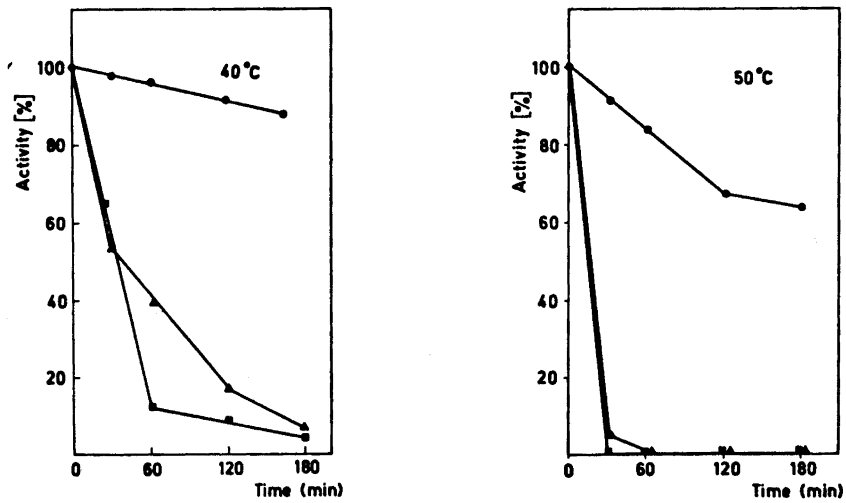

Fig. 4. Thermostability of the peptidases when incubated at 40°C and 50°C, respectively. Aliquots were taken at different time intervals and activity measured. (■), peptidase A (substrate Pro – Gly). (· – · – ·), peptidase B (substrate Pro – Gly). (▲), peptidase C (substrate Ala – Pro).

Usually, the enzyme solutions were kept at 4°C, where peptidases A and B were stable for more than two weeks when merthiolate was added to a final concentration of 0.01 %. The activity loss of peptidase C was about 25 %

after one week under these conditions. The freshly prepared cell extract could also be stored frozen at $-20°$ C or in lyophilized form with only small losses of activity.

E. *Substrate specificity.* Substrate specificity of the three peptidases was determined (Table 4). Peptidase A seems to require N-terminal proline in a dipeptide for activity. (Of all peptides tested, only those were attacked which

Table 4. Specificity of the partially purified peptidases. Activity was measured either quantitatively as described under Methods, or qualitatively with thin layer chromatography or high voltage paper electrophoresis. 100 – 75 % activity + + + +. 75 – 50 % activity + + +. 25 – 5 % activity + +. < 5 % activity +. No activity –. 100 % activity is defined as the specific activity of the particular enzyme preparation against the best substrate investigated.

	Peptidase A	Peptidase B	Peptidase C
Pro – Gly	+ + + +	+ + + +	–
Pro – Leu	+ + +	+ +	–
Poly – Pro	(+)	–	–
Ala – Pro	–	–	+ + + +
Gly – Pro	–	–	+
Leu – Gly	–	+ + + +	–
Gly – Leu	–	+ +	–
Gly – Gly	–	+	–
Ala – Gly			(+)
Ala – Pro – Gly	–	(+)	–
Gly – Pro – Ala	–	–	–
Lys – Pro – Gly	–	–	–
CBZ – Gly – Leu		–	
CBZ – Ala – Pro			–
Ala – Ala – Ala – Ala			+ +
Leu – Gly – Pro – Ala	–	–	–
Gly – Pro – Gly – Leu – Pro	–	–	–
Poly – (Pro – Gly – Pro)	–	–	–

have free proline at the N-terminal end.) Polyproline was attacked very slowly compared to the dipeptides.

Peptidase B has a broad specificity and cleaves a large number of dipeptides. Peptides with blocked terminal amino groups are not hydrolyzed. CBZ-Leu-Gly and CBZ-Gly-Leu are attacked very slowly compared to the controls with free N-terminals.

Peptidase C appears to have a narrow substrate specificity. It hydrolyzes Ala-Pro most rapidly, but also Gly-Pro at a slow rate. More surprisingly, this enzyme also hydrolyzes tetra-Ala, and very slowly Ala-Gly. Here again, a free N-terminal seems necessary, as CBZ-Ala-Pro is resistant to hydrolysis.

None of the peptidases were found to split longer peptides such as Ala-Pro-Gly, Gly-Pro-Ala, or Leu-Gly-Pro-Ala, through low activities could be observed in the crude cell extract toward these peptides.

III. Cultivation of bacteria on different substrates

The enzyme activities of extracts from cells grown in some different media were studied. The basal medium was the mineral salts medium M-63 to which different compounds were added. For these experiments the cells were grown in Fernbach flasks, containing 1 l medium and incubated on a rotary shaker at 30°C, and the cells being harvested at the end of the exponential growth phase. The results are shown in Table 5. Highest activities were found after

Table 5. Specific activities of crude enzyme preparation against peptide substrate after cultivation of bacteria of different substrates.

Substances added to medium M63	Pro – Gly	Ala – Pro
1 % gelatine + 0.1 % casitone	172	33.5
1 % glycerol + 0.01 % biotin	91.2	13.5
0.5 % glucose + 0.01 % biotin	38.7	9.3
1 % glycerol + 0.1 % leucine + 0.01 % biotin	88.2	13.4
1 % glycerol + 0.1 % alanine + 0.01 % biotin	72.4	19.1
1 % glycerol + 0.1 % proline + 0.01 % biotin	151	6.9
1 % glycerol + 0.1 % lysine + 0.01 % biotin	41.5	12.8
1 % glycerol + 0.1 % glutamic acid + 0.01 % biotin	65.5	16.2

growth on gelatin and peptone, but none of the other substances added completely repressed any of the two activities studied. This suggests that the cells form appreciable quantities of enzymes under most culture conditions.

DISCUSSION

Chromatography on DEAE-cellulose or zone electrophoresis show that *Arthrobacter* extracts contain at least three different peptidases.

Peptidase A seems to be similar to a proline iminopeptidase from *E. coli* K. described by Sarid *et al.*[1-2] The latter enzyme also splits longer proline-peptides, but acts only on free N-terminal proline. Davis and Smith[13] have described an iminodipeptidase (prolinase) from swine kidney which does not cleave longer peptides than dipeptides, but also those where proline is substituted by hydroxy-proline. However, peptidase A of *Arthrobacter* does not hydrolyze hydroxyproline peptides.

The proline peptidase from *E. coli* B. described by Yaron[3] acts on the bond between an N-terminal amino acid, followed by a proline residue: A – Pro – B – C . . . Peptidase C cleaves the same type of bond, but acts only on dipeptides. Smith purified an imidodipeptidase (prolidase) from swine kidney,[14] which hydrolyzed C-terminal proline residues. Hydroxyproline could also replace proline. The presence of the imido bond in the X – Pro-peptides would suggest the existence of a specific enzyme for its hydrolysis. The ability of *Arthrobacter* peptidase C to hydrolyze tetra-Ala is not understood, as this is a peptide where only amido bonds occur.

The classification of peptidase B is more difficult. Several unsuccessful attempts were made to fractionate the different activities of this preparation. Furthermore, pH-optimum, stability, and metal activation of the preparation, as examined with both Pro-Gly and Leu-Gly as substrates, revealed no significant differences. These results imply that peptidase B represents a single enzyme with a broad substrate specificity. It is uncertain whether peptidases B and C should be regarded as true metalloenzymes. Both are almost completely inhibited by EDTA, but full activity was not regained when they were incubated with different metal ions. No increase in the enzyme activities over the controls was observed with these metal ions. This may be due to incorrect concentrations of the ions added.

Inhibition of peptidase C does not necessarily indicate thiol groups at the active site. If the system is complex and the enzyme not pure, the inhibition may be due to -SH groups in possible cofactors involved in the system.[15] Another possible way in which thiol reagents can inactivate enzymes in which the -SH group is not part of the active centre, is by steric hindrance.[16-17]

Some peptidases catalyse reactions such as the synthesis and degradation of cell wall components, which could also be one of the physiological functions of these enzymes. [18-23] They are truly intracellular and cannot therefore digest nutrient proteins in the extracellular medium, but could take part in the intracellular turnover of proteins.[24]

Studies on the enzymes *in vitro* and in a purified state do not, however, permit strict conclusions regarding their physiological rate. *In vitro* experiments often reflect only a part of the repertory of the enzyme, and its properties might be considerably changed by the environment in the intact cell.

Simmonds [25] found, in her studies on *E. coli* K 12, the relative peptidase activity toward Leu-Gly and Gly-Leu to be quite different in intact and broken cells. Young intact cells had also higher activity than older ones, while the activity was maintained throughout the aging process when assayed in broken cells.

The three peptidases described here show relatively high stability. They could therefore be of value in protein chemistry, in which there is a need for enzymes which cleave pro-X and X-pro bonds.

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