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GENERAL PROPERTIES AND SUBSTRATE SPECIFICITY OF AN INTRACELLULAR NEUTRAL PROTEASE FROM *STREPTOCOCCUS DIACETILACTIS*

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Abbreviations

FAGLA : Furylacryloyl glycyl L-leucine amide.
BANA : α-N Benzoyl-DL-Arginine *p*-nitroanilide.
SPAA : Succinyl-L-Phenylalanine *p*-nitroanilide.
Z : Benzyloxycarbonyl.
BAEE : N-Benzoyl-L-Arginine ethyl ester.
ATEE : N-acetyl-L-Tyrosine ethyl ester.
Dip-F : Diisopropylfluorophosphate.
PMSF : Phenylmethyl sulfonyl fluoride.
p-CMB : *p*-hydroxymercuribenzoate.

SUMMARY

An intracellular endopeptidase is isolated from *S. diacetilactis*. Its molecular weight, determined by gel filtration on Sephadex G-100, is 49 500 daltons. Using oxidized insulin as substrate, maximal activity is recorded at 45°C with a pH of about 7.0 ; apparent energy of activation is 18 000 cal/mole. The endopeptidase is most stable at temperatures under 37°C. It is totally inactivated by 1 mM of EDTA then reactivated by metal ions (Mn^{++} or Co^{++}). *p*-CMB induces a slight reduction in enzyme activity ; Dip-F is not an inhibitor.

This protease rapidly attacks oxidized insulin, glucagon or α_{s_1}-casein but does not appear to hydrolyze proteins (insulin, ribonuclease, β-lactoglobulin, azocasein), the usual esters, mono- or disubstituted dipeptides or substituted amino acids. Endopeptidase specificity is determined by analyzing the peptides in digests of carboxymethylated β-chain insulin. All the bonds readily cleaving are those involving the α-amino group of hydrophobic residues, *i.e.* X-Leu or X-Phe. This intracellular enzyme is therefore a « neutral microbial metalloenzyme » (EC 3.4.24.4).

INTRODUCTION

Lactic acid streptococci show complex and variable nutritional requirements. Minimal requirements may involve amino-acids, peptides, purines, pyrimidines, vitamins and occasionally fatty acids and elevated CO_2 tension (DEIBEL and SEELEY, 1974). *Streptococcus diacetilactis* in particular has an arginine or phenylalanine requirement (REITER and ORAM, 1962). Therefore the growth of lactic acid streptococci in milk can generally be stimulated by enzymatic hydrolysates of protein (GARVIE and MABBITT, 1956 ; SPECK *et al.*, 1958).

The present work constitutes part of a more general study in progress to determine the mechanism of peptidic stimulation of lactic streptococcus growth in milk. Since these peptides are a particular source of amino-acids (DESMAZEAUD and HERMIER, 1972, 1973), we decided to investigate how they are hydrolyzed by intracellular proteolytic enzymes.

The growth of *S. thermophilus* is highly stimulated by peptides ; we have already characterized an aminopeptidase and dipeptidase (RABIER and DESMAZEAUD, 1973) in this streptococcus as well as a neutral endopeptidase (DESMAZEAUD, 1974) with low proteolytic action. We wished to compare it with the enzyme equipment of *S. diacetilactis*, the growth of which is poorly stimulated by peptides. This study is necessary since information about intracellular proteases in N group streptococci is still very scarce and concerns only *S. lactis*.

Proteolytic activity in *S. diacetilactis* is described in the present paper as well as the general properties of the main proteolytic fraction ; its substrate specificity is determined on the insulin β-chain.

MATERIALS AND METHODS

Materials

The origin of the different products has been described previously (RABIER and DESMAZEAUD, 1973 ; DESMAZEAUD, 1974), except for FAGLA (Monsanto Co), α-N-Benzoyl-DL-Arg-*p*-nitroanilide and Azocasein (Sigma), Succinyl-L-Phe-*p*-nitroanilide (Boehringer). Insulin was oxidized according to the method of SCHRAM *et al.* (1954).

Organism.

Streptococcus diacetilactis : strain CNRZ 267.

This bacterium was maintained by subculture on sterile skim-milk and preserved by freezing at —30°C.

Cell cultures.

350 g of moist cells were obtained in a fermentor from 75 liters of a previously described culture medium of papain-hydrolyzed milk (VALLES and MOCQUOT, 1968) (pH maintained at a constant value of 6.5). The bacteria were collected after the exponential growth phase as preliminary results had shown that proteolytic activity was maximum at that culture time.

Measurement of proteins and enzyme activities.

Proteins.

Protein content was determined according to LAYNE (1957) and LOWRY *et al.* (1951) ; serum albumin was used as a control.

Proteolytic activity was determined at 37°C by measuring :

(i) liberated α-amino groups after ninhydrin coloration according to MOORE and STEIN (1954) on the following substrates : insulin, oxidized-insulin, ribonuclease A, oxidized-ribonuclease A, α-S_1-casein, glucagon, β-lactoglobulin, serum albumin. The reaction mixture contained : 0.8 ml substrate (0.03 p. 100 concentration) in 0.1 M sodium phosphate buffer, pH 7.0 ; 0.2 ml of a suitable dilution of enzyme preparation. The substrate used was generally oxidized-insulin ; (ii) absorbance variation at 440 nm on azocasein substrate according to CHARNEY and TOMARELLI (1947) ; (iii) liberated α-amino group on Z-Gly-Leu-NH_2, Z-Gly-Phe-NH_2 according to MORIHARA *et al.* (1968) ; (iv) absorbance variation at 345 nm on FAGLA according to KEAY and WILDI (1970) ; at 410 nm on BANA, SPAA according to ERLANGER *et al.* (1961) ; at 253 nm on BAEE and at 237 nm on ATEE according to SCHWERT and TAKENAKA (14).
Dipeptidase activity was determined at 37°C by measuring liberated L-Leu on Leu-Leu substrate after ninhydrin coloration. The reaction mixture contained : 0.8 ml substrate (0.5 mM) in 0.1 M sodium phosphate buffer, pH 7.0 ; 0.2 ml of a suitable dilution of enzyme preparation.
Aminopeptidase activity was assayed at 37°C on Leu-*p*-nitroanilide substrate according to RONCARI and ZUBER (1969).
Carboxypeptidase activity was assayed at 37°C on Z-Gly-AA_2 (AA_2 = Ala, Phe, Lys or Arg) and Z-Glu-Tyr after ninhydrin coloration according to MORIHARA *et al.* (1968).

Polyacrylamide gels.

The polyacrylamide gels (7 p. 100 acrylamide) were prepared according to the method of ORNSTEIN (1964) and DAVIS (1964). After electrophoresis, the gels were stained with Coomassie Blue according to CHRAMBACH *et al.* (1967) or cut consecutively into discs of equal thickness (1.5 mm) and directly used for assay of peptidase or proteolytic activity.

Substrate specificity.

Digestion of B-chain carboxymethylated-insulin.

Incubation was done at 37°C in 0.05 M sodium phosphate buffer (pH 7.5) for 10 minutes with a ponderal enzyme/substrate ratio of 1/50 (experiment A) and for 6 hours with an E/S ratio of 1/25 (experiment B).

Isolation and purification of hydrolysate peptides.

Peptide fragments were isolated on Bio-Rad AG 50 W × 2 type of cation exchange resin prepared according to the method of SCHROEDER (1967). At the final step it was equilibrated with pyridine-acetic acid-water buffer, pH 3.10 and a pyridine concentration of 0.1 N (experiments A and B_2) or 0.2 N (experiment B_1). The peptides were eluted by linear gradients of pyridine molarity, pyridine was distilled before utilization. Detection of the peptides after alkaline hydrolysis has been described elsewhere (DESMAZEAUD, 1972). In addition, some of these peptides were purified by high voltage electrophoresis or paper chromatography according to techniques already described (DESMAZEAUD and HERMIER, 1972 ; DESMAZEAUD, 1974).

Quantitative amino acid composition and concentration of purified peptides.

Determination was carried out using a Multichrome autoanalyser (Beckman) after hydrolysis by tridistilled HCl, 6 N, in vacuum sealed tubes for 24 h at 115°C. In ambiguous cases N-terminal amino acids of some peptides were determined by dansylation, and then by bidimensional chromatography on micropolyamide sheets (Schleicher-Schüll) according to the method of HARTLEY (1970).

RESULTS

Isolation and purification of protease A_1

Step 1. Preparation of the extract — Elimination of nucleic acids.

350 g of cells were washed twice in 1 500 ml of 0.05 M sodium phosphate buffer (pH 7.0). The cells were resuspended in 1 500 ml of the same buffer and crushed in a Manton-Gaulin homogenizer, type 15 M/8 TA (APV-France), under a pressure

of 8 000 psi. The cell suspension was then centrifuged at 10 000 g for 1 h at 4°C. The supernatant (2 000 ml) was preserved and the nucleic acids hydrolyzed by the addition of ribonuclease (25 µg/ml) and deoxyribonuclease (0.1 µg/ml) in presence of $MgCl_2$, 6 H_2O, 0.8 mM and then incubated for 45 min at 27°C. They were precipitated with 10 g of $MnSO_4$; after 1 h at 4°C the precipitate was eliminated by centrifugation at 10 000 g for 10 min and the supernatant recovered. Finally, after concentration to 124 ml in a Diaflo cell (Amicon) using a UM 10 membrane, the extract was chromatographed on a Sepharose 6 B gel column (4 cm × 96 cm) (Pharmacia), equilibriated with 0.05 M sodium phosphate buffer (table 1). The proteolytic extract (PE) obtained in this way did not contain any dosable nucleic acids according to LAYNE (1957). It contained dipeptidase activity, but did not hydrolyze amino- or carboxypeptidase substrates.

TABLE I

Purification of protease A_1 from S. diacetilactis
Purification de la protéase A_1 de S. diacetilactis

	Volume (ml)	Activity (Units/ml)	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)	Purification (fold)
Step. 1							
a) *Elimination of nucleic acids* ...	2 000	17.5	35 000	28 000	1.25	100	1
b) *After Sepharose 6 B.* PE extract	1 600	15.75	25 200	8 000	3.15	72	2.5
Step 2. DEAE-cellulose chromatography							
Protease A	560	11.40	6 400 (49.2 %)**	500	12.8	37*	20.8*
Protease B	2 800	2.35	6 600 (50.8 %)**	900	7.33	37*	11.5*
Step 3. ECTEOLA-cellulose chromatography (from Protease A)							
Protease A_1	180	15	2 700	56.5	47.8	15.6*	77.7*
Protease A_2	150	4	600	33	18.2	3.5*	29.6*
Step 4. Sephadex G-100 chromatography (from protease A_1) ...	38	44.2	1 680	22	76.34	9.7*	124.1*

* Values calculated from percentages indicated for under **.

Step 2. DEAE-cellulose chromatography.

The PE extract (8 g of protein) in 0.05 M sodium phosphate buffer was absorbed on a DEAE-cellulose column equilibriated with the same buffer. The column was then washed with the buffer and the proteins eluted successively with a gradient

of sodium phosphate buffer. The protease A was thus eluted at the beginning of the 0.2 M phosphate gradient. Another proteolytic fraction (protease B) overlapping dipeptidase activity was also separated in this way (fig. 1) ; these enzymes will be described in another article. The A extract did not show any aminopeptidase (on Leu -*p*-nitroanilide) or carboxypeptidase activity (on Z-Glu-Tyr). The active fractions, corresponding to protease A and representing 500 mg of protein, were combined and dialyzed against 0.05 M sodium phosphate buffer at pH 7.0 (table 1). The contribution of this protease A to the total proteolytic activity of the initial extract (49.2 p. 100) was calculated from this step and allowed us to estimate yield and purification rates.

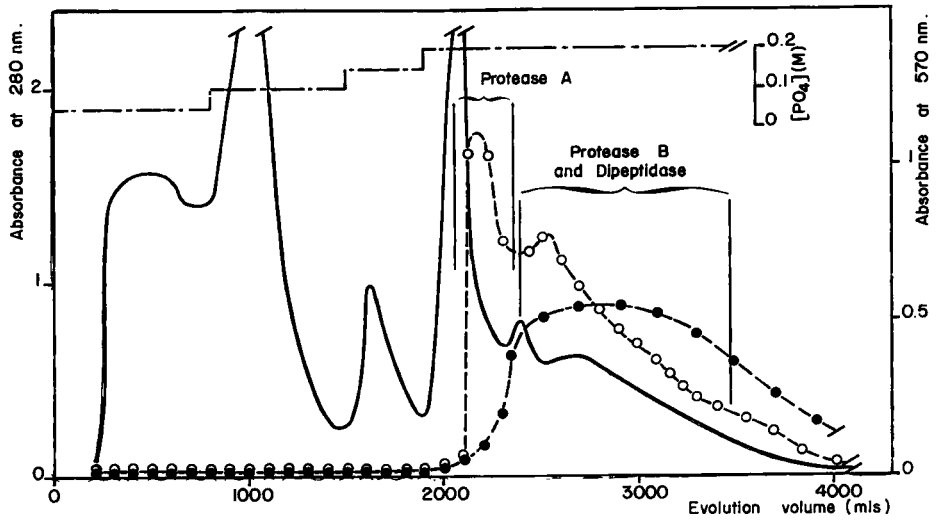

FIG. 1. — *DEAE-cellulose chromatography of proteases and dipeptidase from* S. diacetilactis

Chromatographie sur DEAE-cellulose des protéases et de la dipeptidase de S. diacetilactis

The column was 40 cm high with a diameter of 2.5 cm. The DEAE-cellulose DE-23 (Whatman) was equilibrated with 50 mM sodium phosphate (pH 7.0) ; its flow rate was 40 ml/h. The nucleic acid-free extract of 8 g protein in 50 mM phosphate buffer was absorbed on the column. Proteins were eluted by sodium phosphate gradients at pH 7.0. 10 ml fractions were collected. Proteolytic activity (o — — o) was determined by hydrolysis of oxidized insulin, dipeptide-hydrolase activity (• — — •) by hydrolysis of Leu-Leu then in both cases by measuring liberated α-amino group (absorbance at 570 nm) as indicated in « Materials and Methods ». Protein elution was followed by measuring absorbance at 280 nm (———).(——— - ———) indicates buffer molarity.

Step 3. ECTEOLA-cellulose chromatography.

After being concentrated 15 times on a Diaflo UM 10 membrane, the protease A was applied to an ECTEOLA-cellulose (Sigma) column prepared with 0.01 M sodium phosphate buffer. After washing the column with the same buffer, the protease A was eluted with a linear phosphate buffer concentration gradient at pH 7.0. Thus, the main protease, called protease A_1, was eluted at a phosphate concentration of 0.025 M. No more exopeptidase activity was found on Leu-Leu (fig. 2). The minor protease, called protease A_2, will be described in another article. Protease A_1 represented 56.5 mg of protein (table 1).

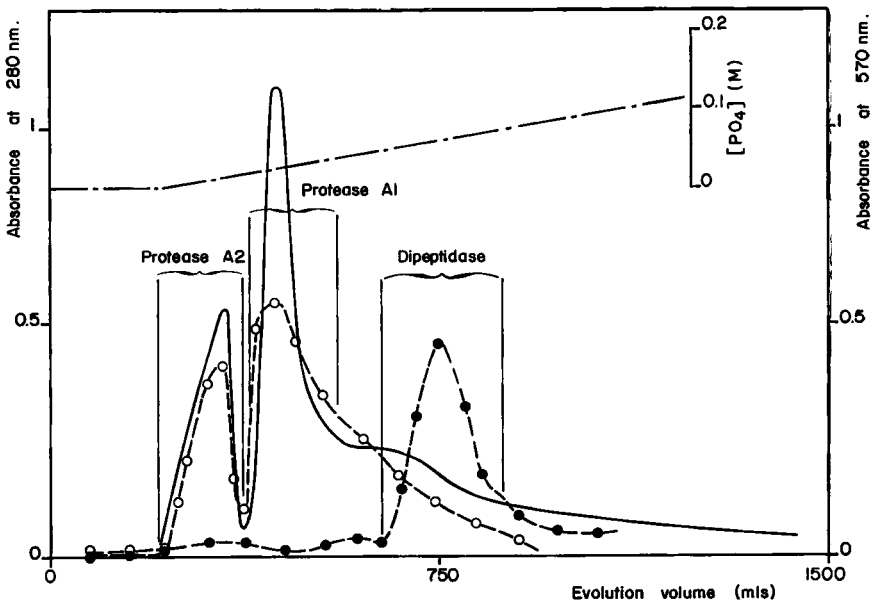

FIG. 2. — *ECTEOLA-cellulose chromatography of proteases from* S. diacetilactis

Chromatographie sur ECTEOLA-cellulose des protéases de S. diacetilactis

The column was 30 cm high with a diameter of 2 cm. ECTEOLA-cellulose was equilibrated with 10 mM sodium phosphate (pH 7.0) ; its flow rate was 35 ml/h. 9 ml fractions were collected. Fractions with high proteolytic activity (Protease A) eluted from the DEAE-cellulose column (fig. 1) were pooled and concentrated on Diaflo UM10 membrane. This extract was previously equilibrated by dialysis in the same buffer and then absorbed on the column. Proteins were eluted by linear sodium phosphate buffer gradient prepared with 1 000 ml of 10 mM sodium phosphate buffer (pH 7.0) from the mixing chamber and an equal volume of 0.2 M sodium phosphate buffer (pH 7.0) from the container. Proteolytic activity (o — — o) was determined by hydrolysis of oxidized-insulin, dipeptide hydrolase activity (• — — •) by hydrolysis of Leu-Leu and measurement of the liberated α-amino group (absorbance at 570 nm) as indicated in « Materials and Methods ». Eluted proteins were measured at 280 nm (——) ——·—— : indicates buffer molarity.

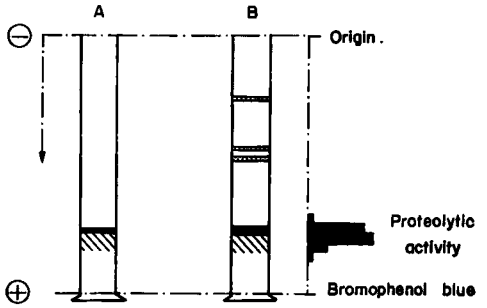

FIG. 3. — *Polyacrylamide gel electrophoresis of protease* A_1

Électrophorèse en gel de polyacrylamide de la protéase A_1

A = experiment with 35 µl of protease A_1.
B = experiment with 130 µl of protease A_1.
Proteolytic activity was detected on experiment B

Step 4. Gel chromatography and polyacrylamide gel electrophoresis.

The active fractions of protease A_1 were concentrated 18 times on a Diaflo UM 10 membrane and filtered on a Sephadex G-100 (Pharmacia) column (2.5 × 90 cm) equilibriated with 0.05 M sodium phosphate buffer at pH 7.0. Protease A_1 was eluted with the same buffer at a rate of 15 ml/h leading to obtention of 22 mg purified protease (table 1). This enzyme could be purified further by polyacrylamide gel electrophoresis at pH 8.5 ; the extract was still heterogeneous after passage on Sephadex G-100.

However, after cutting the polyacrylamide gel the protease was eluted by crushing the small gel discs in 0.05 M sodium phosphate buffer. Furthermore, this step confirmed that only one zone of proteolytic activity was present on the oxidized-insulin and that protease A_1 was not contaminated by any exopeptidase activity (fig. 3). This enzyme was thus considered as sufficiently pure for study of its general properties.

Molecular weight

Apparent molecular weight was estimated according to the method of ANDREWS (1964) with a Sephadex G-100 column (fig. 4). The apparent molecular weight of the protease was 49, 500 daltons (with reference to that of trypsin, pepsin, serum-albumin).

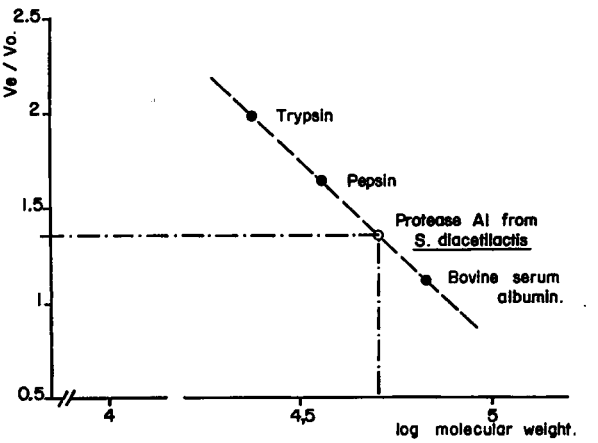

FIG. 4. — *Estimation of molecular weight of protease A_1 on Sephadex G-100*

Estimation du poids moléculaire de la protéase A_1 par chromatographie sur gel Sephadex G-100

The column was 90 cm high with a diameter of 2.5 cm. The Sephadex G-100 was equilibrated with 50 mM sodium phosphate (pH 7.0). Flow rate was 15 ml/h. The protease A_1 was eluted with the same buffer. ·—·—· indicates the value of the logarithm of molecular weight of the protease A_1.

$$Ve/Vo = \frac{\text{elution volume of the protein}}{\text{elution volume of Blue Dextran 2000}}$$

Effect of pH

Maximum activity was obtained at pH 7.0. Fifty per cent of this activity was measured at pH 5.6 or pH 8.0 (fig. 5). The enzyme was quite stable over the pH range 6.5-7.0. Thirty or 35 p. 100 of the protease was readily inactivated at pH 5.5 or pH 8.0, respectively, after 30 min at 37°C (fig. 5 A).

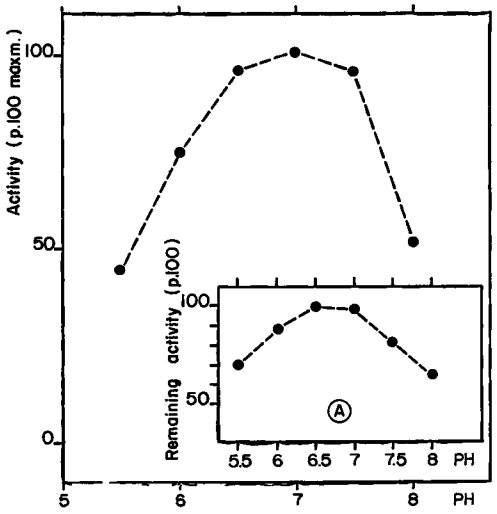

FIG. 5. — *Activity and stability* (Part A) *of protease A_1 as a function of pH*

Activité et stabilité (partie A) *de la protéase A_1 en fonction du pH*

Proteolytic activity of protease A_1 (step 4) on oxidized-insulin was tested in pH range from 5.6 to 8.0 in 0.1 M sodium phosphate buffer. The activity was measured at 37°C for 30 min and expressed as per cent of maximum activity.

Part A. Effect of pH on the stability of the protease A_1. The enzyme was incubated for 30 min at 37°C in 10 mM sodium phosphate buffer adjusted to the desired pH. The remaining activity was estimated at pH 7.0 as indicated above and expressed as a percentage of the control.

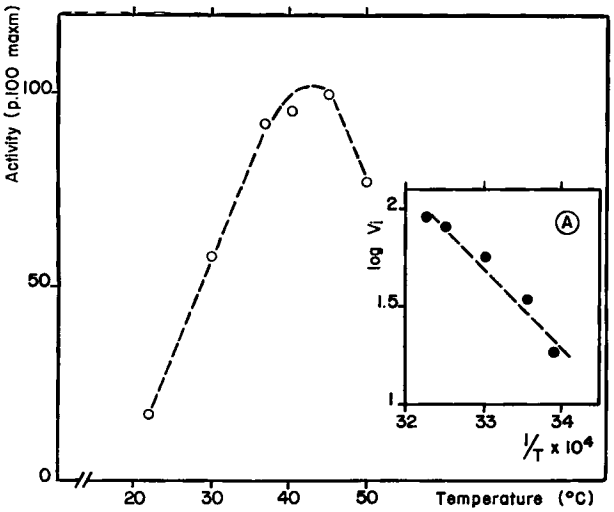

FIG. 6. — *Effect of incubation temperature on proteolytic activity A_1*

Influence de la température d'incubation sur l'activité de la protéase A_1

Proteolytic activity of protease A_1 (step 4) on oxidized insulin was tested in temperatures ranging from 22 to 50°C. The activity was estimated after 20 min incubation at pH 7.0 and expressed as per cent of maximum activity.

Part A : Arrhenius plot (MOELWYN-HUGHES, 1950) made from the results of figure 6.

Effect of temperature

The maximum temperature for oxidized-insulin hydrolysis was 45°C at pH 7.5 after 20 min (fig. 6) ; its apparent energy of activation was 18 000 cal/mole (fig. 6 A).

The purified enzyme (step 4) retained its activity after storage over several months at — 20°C. It could be kept for 6 h at 30°C without losing its activity, but was rapidly inactivated at higher temperatures. Twenty per cent of its activity was lost after 30 min at 40°C (fig. 7) and 81 p. 100 after 30 min at 45°C. The protease was readily inactivated at 50°C (fig. 7).

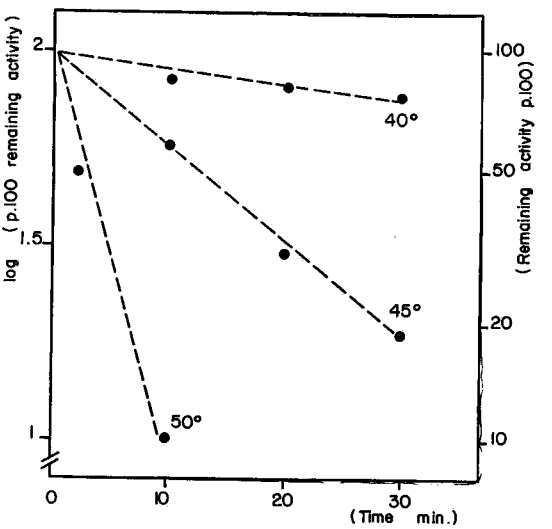

FIG. 7. — *Thermal denaturation of protease A_1*

Dénaturation thermique de la protéase A_1

Protease A_1 (step 4) was incubated at pH 7.0 in 0.01 M sodium phosphate buffer at 40, 45 and 50°C for 5, 10, 20 and 30 min. The remaining activity was estimated at pH 7.0 for 20 min at 37°C on oxidized-insulin and expressed as a percentage of the control on a logarithmic plot.

Effect of various inhibitors and metal-ion requirement

Seryl inhibitors such as Dip-F and PMSF showed no inhibition. The sulfhydryl inhibitor mM *p*-CMB reduced activity to 40 p. 100 (table 2), but 0.1 mM had no effect on the protease. There is no seryl residue therefore in the active site of protease A_1, and sulfhydryl residues are not very important for proteolytic activity.

Addition of 0.01, 0.1 mM EDTA to the reaction mixture reduced the hydrolysis rate of oxidized insulin to 61 and 35 p. 100, respectively, as compared to the controls. Addition of mM EDTA or o-phenanthroline completely inhibited activity (table 2). This inhibition can be fully reversed after dialysis by addition of Mn^{++} or after dialysis against Co^{++} or Mn^{++}. Metal ions such as Zn^{++} or Ca^{++} can reactivate the protease to a certain extent (table 2). The results clearly indicate that the enzyme requires a divalent ion for its activity, but this can only be shown after pre-incubation in the presence of EDTA, probably because in the purified enzyme the cation

is still tightly bound. Indeed, protease catalysed oxidized-insulin degradation when exogenous metal ions were absent in the reaction mixture and the addition of Ca^{++}, Mg^{++}, Co^{++}, Zn^{++} or Mn^{++} had no stimulatory effect on proteolysis rate.

TABLE 2

Effects of protease inhibitors and divalent cations

Effets des inhibiteurs et des cations divalents

The enzyme was pre-incubated 15 min at 37°C with inhibitor in 0.05 M Tris HCl, pH 7.5. The remaining proteolytic activity of protease A_1 was measured on oxidized-insulin at 37°C during 15 min in 0.05 M Tris-HCl buffer pH 7.5 and is expressed as a percentage of the control.

Pre-incubation 15 min 37°C	Incubation 15 min 37°C	Proteolytic activity as % of control
	1 mM Dip-F	100
	1 mM PMSF	100
	0.1 mM *p*-CMB	95
	1 mM *p*-CMB	40
	0.01 mM EDTA	61
	0.1 mM EDTA	35
	1 mM EDTA	0
	1 mM *o*-phenanthroline	0
a)		
1 mM EDTA followed by dialysis (16 h, 4°C) against 0.05 M *Tris*-HCl, pH 7.5 [1]		9
as (*a*)	1 mM Ca^{++}	0
as (*a*)	1 mM Mg^{++}	0
as (*a*)	1 mM Co^{++}	26
as (*a*)	1 mM Zn^{++}	74
as (*a*)	1 mM Mn^{++}	112
b)		
1 mM EDTA	Dialysis (16 h, 4°C) against 1 mM divalent cations in 0.05 M *Tris*-HCl pH 7.5	
as (*b*)	Mg^{++}	0
as (*b*)	Ca^{++}	61
as (*b*)	Zn^{++}	92
as (*b*)	Mn^{++}	109
as (*b*)	Co^{++}	112

[1] Without dialysis we have not reversed this inhibition by direct addition of divalent cations.

Substrate specificity

Hydrolysis of B-chain carboxymethylated-insulin.

After 10 min of hydrolysis (experiment A) 9 fractions were separated on Bio-Rad AG-50 W × 2 (fig. 8 A). The peptides were purified and we located them on the B-chain of insulin by their quantitative amino acid composition (fig. 9). Five

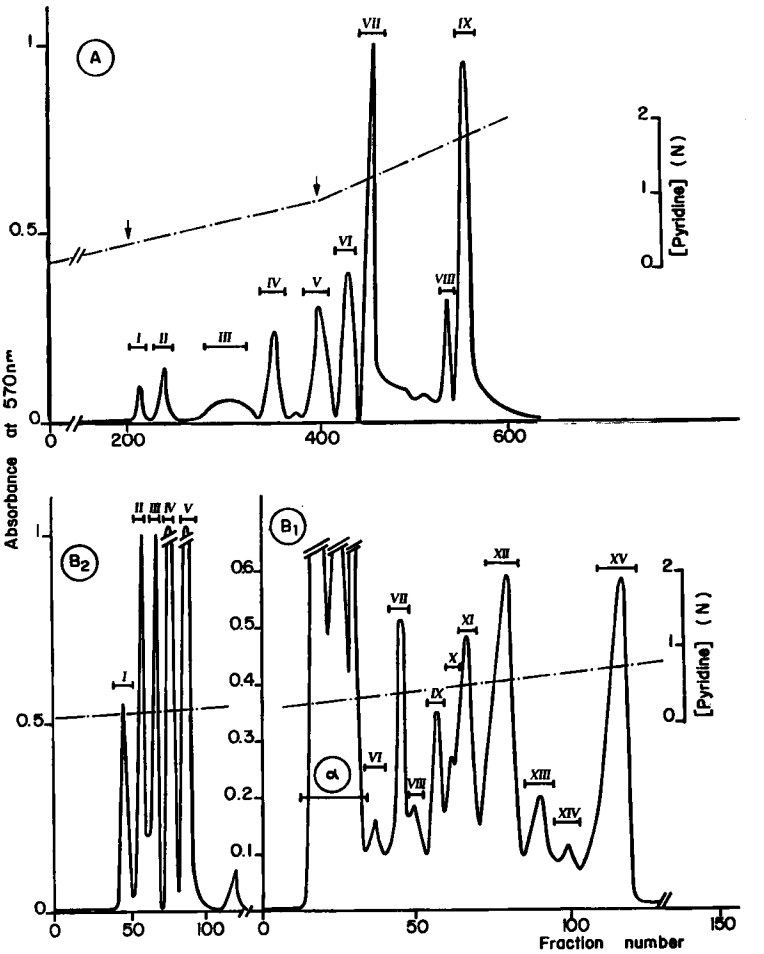

FIG. 8. — *Column chromatography of the enzymic digests of carboxymethylated B-chain insulin*

Chromatographie des hydrolysats de la chaîne B carboxyméthylée de l'insuline

Part A. The digests of experiment A (50 mg), as indicated in « Materials and Methods », were applied to a column of Bio-Rad AG-50 W × 2 (Aminex 200-325 mesh) (50 × 1 cm) equilibrated with the starting buffer 0.1 N pyridine-acetic acid-water (8-250-742) pH 3.1. The peptides were eluted by three linear pyridine-acid acetic-water buffer gradients, as follows by ↓. The first one : from starting buffer (500 ml) to 0.3 N pyridine-acetic acid-water (500 ml) 24-100-876, pH 3.8. The second one : from 0.3 N pyridine-acetic acid buffer (500 ml) to 0.9 N pyridine-acetic acid-water (500 ml) 72-100-828, pH 4.65. The third one : from 0.9 N pyridine-acetic acid-buffer (500 ml) to 2 N pyridine-acetic acid-water (500 ml) 161-143-696, pH 5.0.

Part B_1. The digest of experiment B (100 mg), as indicated in « Materials and Methods », was applied to a column of Bio-Rad AG-50 W × 2 (Aminex 200-325 mesh) (65 × 1 cm) equilibrated with a starting buffer 0.2 N pyridine-acetic acid-water (16-279-705) pH 3.1. The peptides were eluted with a linear pyridine-acetic acid buffer gradient made from 0.2 N pyridine-acetic acid-water starting buffer pH 3.1 (1 000 ml) to 2 N pyridine-acetic acid-water (1 000 ml) 161-143-696, pH 5.0.

Part B_2. This was a rechromatography of fraction α of B_1 on the same column equilibrated with a starting buffer 0.1 N pyridine-acetic acid, pH 3.1. The peptides were eluted with a linear pyridine-acetic buffer gradient made from 0.1 N pyridine-acetic acid starting buffer, pH 3.1 (500 ml) to 0.5 N pyridine-acetic acid-water (500 ml) 40-30-930, pH 5.0. For these chromatographies, the flow rate was adjusted to about 25 ml/hour, the temperature to 40°C and a 5 ml volume was collected from each tube. Ninhydrin was determined with 0.5 ml portions (absorbance at 570 nm ——) after alkaline hydrolysis by 1.25 N NaOH, 2 h at 100°C - ——·——- : indicates pyridine molarity. The lines above the peaks indicate the fractions which were pooled for further analysis.

peptide bonds were broken during the first steps of the hydrolysis. Hydrolysis of the Tyr_{16}-Leu_{17} and Phe_{25}-Tyr_{26} bonds was strong, that of the Ala_{14}-Leu_{15} and His_{10}-Leu_{11} weak and that of Gly_{23}-Phe_{24} very weak (fig. 9 and 10).

Fifteen fractions were separated on Bio-Rad AG-50 W × 2 resin after 6 h of hydrolysis. The overlapped peaks I-V (α fraction of fig. 8 B_1) were separated by a second chromatography with a low concentration gradient (fig. 8 B_2). After additional fractionation and peptide purification by paper chromatography or electrophoresis, their quantitative amino acid composition and terminal-NH_2 groups were determined. When these peptides were located on the insulin B-chain (fig. 9) the rupture of new peptide bonds appeared such as His_5-Leu_6, Pro_{28}-Lys_{29}, Lys_{29}-Ala_{30}. Hydrolysis of the Phe_1-Val_2, Phe_{24}-Phe_{25} and Tyr_{26}-Thr_{27} bonds seemed very weak (figs. 9 and 10). Moreover, previously obtained hydrolysis of the bonds Tyr_{16}-Leu_{17}, Gly_{23}-Phe_{24} and His_{10}-Leu_{11} was considerably increased (figs. 9 and 10). Thus, the first hydrolyzed peptide bonds exhibited an apolar residue (Leu and Phe) bound by its amino group. The same applied to the other peptide bonds split after prolonged hydrolysis. These had Leu, Phe, Ala or Tyr in amino position except for the slightly hydrolyzed Pro_{28}-Lys_{29} bond or very slightly hydrolysed Tyr_{26}-Thr_{27} bond, both representing only 9 p. 100 of the total hydrolysis (fig. 10 B).

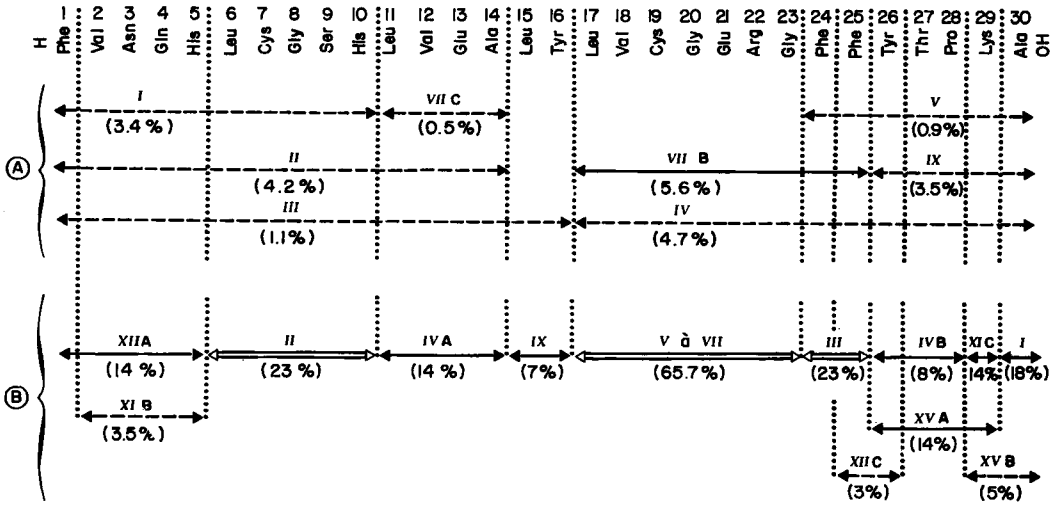

FIG. 9. — *Localization and yields of the peptide fragments of the carboxymethylated B-chain insulin digested by protease A_1*

(μmoles of peptides per μmoles B-chain)

Localisation et rendements des peptides obtenus après hydrolyse de la chaîne B de l'insuline carboxyméthylée, par la protéase A_1

(μmoles de peptides par μmoles de chaîne B)

The designations I to XV of the peptide fragments correspond to the peak numbers in the respective chromatograms of figure 8.

Part A = for a short time;
Part B = for a long time as indicated in « Material and Methods ».

⟸⟹ = indicates peptide fragments in yields above 20 p. 100;
⟵⟶ = indicates peptide fragments in yields between 20 p. 100 and 5 p. 100;
⟵ - - ⟶ = indicates peptide fragments in yields below 5 p. 100.

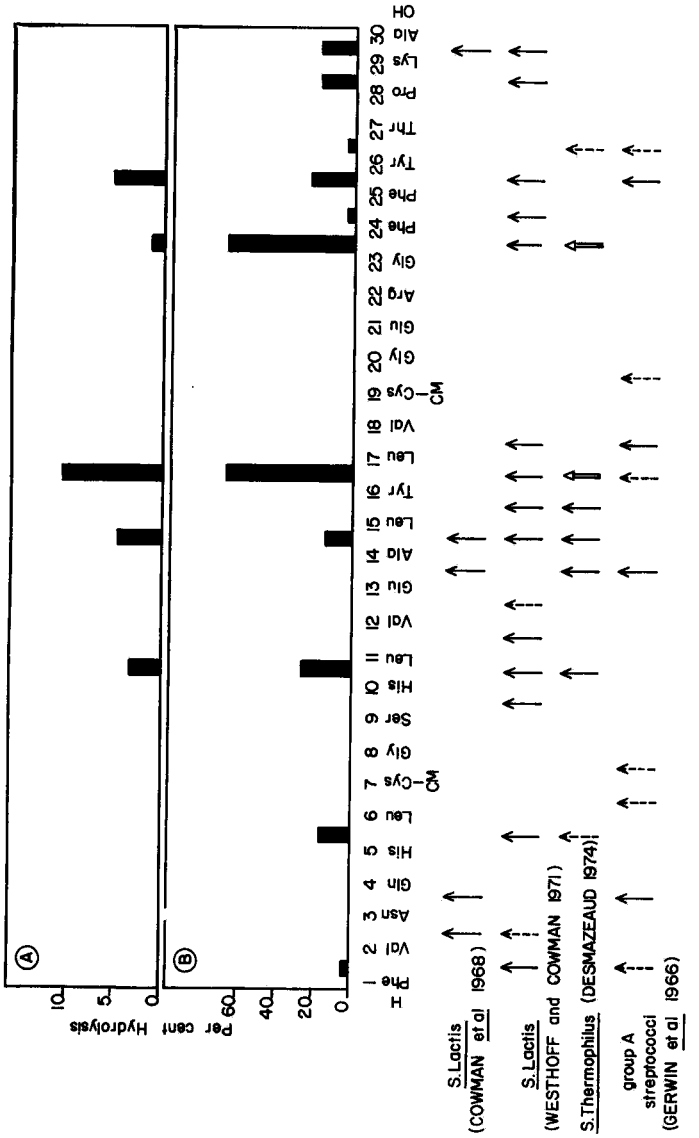

FIG. 10. — *Site of cleavage of carboxymethylated B-chain insulin by protease A_1 of* S. diacetilactis. *Comparison of its specificity with that of various proteinases from* Streptococcus *genus bacteria*

Liaisons hydrolysées par la protease A_1 de S. diacetilactis *dans la chaîne B carboxyméthylée de l'insuline. Comparaison de sa spécificité avec celles des protéases de bactéries du genre* Streptococcus

The degree of hydrolysis of peptide bonds is indicated as a per cent of the hydrolysis of B-chain insulin. *Part A* : for a short time (experiment A as indicated in « Materials and Methods ») ; *Part B* : a long time (experiment B as indicated in « Material and Methods »). Peptide concentrations were summed up on both sides of the cleaved peptide bonds (fig. 9). We used the largest sum as the measure of the cleavage since some peptides could have escaped detection.

Hydrolysis of proteins and synthetic substrates.

In addition to the oxidized-insulin, only glucagon and α_{s1}-casein were hydrolyzed at a satisfactory rate. Isoelectric whole casein, azocasein or oxidized-ribonuclease on the one hand, and disubstituted Z-Gly-Leu-NH_2 dipeptide on the other, were only very slightly hydrolyzed (table 3) ; neither did the protease hydrolyse native proteins such as insulin, ribonuclease, β-lactoglobulin or serum-albumin (table 4). Disubstituted dipeptides, such as Z-Gly-Phe-NH_2 containing a peptide bond sensitive to hydrolysis or FAGLA containing a Leu residue in amino position, were not hydrolyzed either (tables 3 and 4).

TABLE 3

Proteolytic degradation of various protein and peptide substrates. Comparison with the action of thermolysin

Hydrolyse de différents peptides et protéines. Comparaison avec l'action de la thermolysine

The reactions were carried out at pH 7.0 in 0.1 N sodium phosphate buffer at 37°C, with various time intervals up to 6 hours. Specific activities were calculated on an arbitrary enzyme unit (0.01 absorbance unit/min) and expressed as a percentage of the hydrolysis of oxidized-insulin by protease A_1.

Substrate	Specific activity as % hydrolysis of oxidized-insulin	
	Protease A_1 of *S. diacetilactis*	Thermolysin
Oxidized-insulin	100	923
Glucagon	92	3 066
α_{s1}-casein	15	8 461
Oxidized ribonuclease A ...	10	1 384
Azocasein	1.4	318
Z-Gly-Leu-NH_2	1.4	205
FAGLA	0	55

TABLE 4

Substrate non-hydrolyzed by protease A_1 of S. diacetilactis

Substrats non hydrolysés par la protéase A_1 de S. diacetilactis

Reactions were carried out at pH 7.0 in 0.1 N sodium phosphate buffer at 37°C during 6 h with various enzyme concentrations (maximum enzyme/substrate concentration used : 1/10,W/W).

	Z-Gly-Phe-NH_2
Leu-*p*-nitroanilide	Insulin
Leu-Leu	Ribonuclease A
BAEE, BANA	β-lactoglobulin
ATEE, SPAA	Serum albumin
Z-Gly-AA_2 (AA_2 = Ala, Phe, Lys, Arg)	
Z-Glu-Tyr	

Taking oxidized-insulin hydrolysis as a reference, the *S. diacetilactis* protease was definitely less active than thermolysin on these different substrates of the neutral proteases. The hydrolysis rate of azocasein, Z-Gly-Leu-NH_2 and α_{s1}-casein was 227, 146 and 564 times lower, respectively (table 3).

S. diacetilactis protease was not active either on the aminopeptidase substrates (Leu-*p*-nitroanilide or Leu-Leu), the carboxypeptidase substrates (Z-Glu-Tyr, Z-Gly-AA_2) or on substrates of trypsin — or chymotrypsin — like enzymes (BAEE, BANA or ATEE, SPAA) (table 4).

Therefore, this protease is obviously of the « aminoendopeptidase » type (MILLET and ACHER, 1969) and requires a substrate with a minimum chain length and an appropriate configuration to be active.

DISCUSSION

The protease A_1 secreted by *S. diacetilactis* has been purified about 124 times. This seems a fairly good purification considering that the extract contained a contamination visible after gel electrophoresis. The degree of purification is not exact; concentration of protease A_1 in cellular extract could not be fully determined as proteases B and A_2 hydrolyzed the same substrate.

The diffuse zone observed on gel electrophoregrams in front of the main colored band could be produced by an association-dissociation phenomenon. This reaction has been studied with a *S. lactis* intracellular proteinase (COWMAN and SWAISGOOD, 1966).

The protease of *S. diacetilactis* is strictly of an « aminoendopeptidase » type and exhibits optimum pH at neutrality. Its activity is inhibited by EDTA but not by Dip-F. The characteristic properties of this enzyme class it in the group of metal chelator-sensitive neutral proteases of microbial origin (EC. 3.4.24.4) (MATSUBARA and FEDER, 1971). Moreover, its specific action on the insulin B-chain is similar to that of megateriopeptidase (MILLET and ACHER, 1969) or the neutral protease of *B. thermoproteolyticus* (MORIHARA and TSUZUKI, 1966). It is also close to that of *S. thermophilus* (DESMAZEAUD, 1974) which, like the *S. diacetilactis* protease, hydrolyzes the Tyr_{16}-Leu_{17} and Gly_{23}-Phe_{24} bonds very strongly without hydrolyzing Phe_{24}-Phe_{25}; this is contrary to most of the other neutral microbial proteases (MATSUBARA and FEDER, 1971). The very weak hydrolysis of terminal NH_2-Phe of the insulin B-chain has also been obtained by the metallo-proteases of *Bacillus subtilis* var. *amylosaccharititicus* (TSURU *et al.*, 1967), *Streptomyces griseus* (MORIHARA *et al.*, 1968) or *Aspergillus oryzae* (MORIHARA *et al.*, 1968). It was also shown that the neutral proteases of *B. thermoproteolyticus* (MORIHARA and TSUZUKI, 1966 *a*), *Pseudomonas aeruginosa* (MORIHARA and TSUZUKI, 1966 *b*) or *A. oryzae* (MORIHARA *et al.*, 1968) released terminal COOH-Ala.

Among the intracellular proteases of the genus *Streptococcus* (fig. 10), that of *S. diacetilactis* is very similar to *S. thermophilus* (DESMAZEAUD, 1974). In fact, they are both metal chelator-sensitive neutral proteases of similar specificity having a molecular weight of about 40 000 daltons. These proteases, and in particular the

S. diacetilactis protease, are also very sensitive to heat denaturation. This property clearly differentiates them from an intracellular protease of *S. durans* (WALLACE and HARMON, 1970) which is not destroyed by heat treatment at 97°C for 60 min. However, the protease of *S. diacetilactis* exhibits much higher apparent activation energy than that of *S. thermophilus* (DESMAZEAUD, 1974).

This enzyme is very different from two intracellular proteases found in another *Streptococcus, S. lactis*, of the same serological N group. These proteases are not metallo-enzymes. On the other hand, the substrate specificity of the neutral *S. diacetilactis* protease is much narrower than that of the mutant strain of *S. lactis* (WESTHOFF and COWMAN, 1971) which is able to hydrolyze 16 peptide bonds of the insulin B-chain (fig. 10) as well as the non-substituted dipeptides. *S. diacetilactis* protease is also very different from that of the wild strain of *S. lactis* (COWMAN *et al.*, 1968) which shows narrow specificity towards peptide bonds containing an Asn, Glu or Ala residue. Lastly, it is entirely different from the intracellular protease of a *Streptococcus* of the serological A group (GERWIN *et al.*, 1966). This is an enzyme with a sulfhydryl group having a substrate specificity corresponding to the model of BERGER and SCHECHTER (1970).

The strong interaction between the cations and the intracellular protease of *S. diacetilactis* is analogous to that studied in the cytoplasmic protease of *E. coli* (REGNIER and THANG, 1975) which can be reactivated by Mn^{++} ions after inhibition by 1 mM EDTA.

Like the intracellular protease of *S. thermophilus* (DESMAZEAUD, 1974) that of *S. diacetilactis* is incapable of hydrolyzing short-chain substrates or non-denaturated proteins. Thus, the activity of these intracellular microbial enzymes depends on a particular peptide chain length or the spatial configuration of the substrate. Because of this property there is a marked difference between these intracellular neutral proteases and the exocellular neutral microbial proteases which strongly hydrolyze non-denaturated proteins or disubstituted dipeptides. Consequently, the *S. diacetilactis* protease is rather similar to the cytoplasmic metalloprotease of *E. coli* (REGNIER and THANG, 1975) exhibiting a highly limited specificity. A protease presenting this characteristic has also been isolated from the rabbit kidney (KERR and KENNY, 1974). Proteins, including insulin, are not or only slightly hydrolyzed by this endopeptidase. However, the insulin B-chain having bonds sensitive to neutral proteases is strongly hydrolyzed.

The function of protease A_1 in *S. diacetilactis* metabolism is unknown. Owing to its restricted specificity, protease A_1 may be involved in protein or peptide degradation, and act in conjunction with another protease or peptidase. This suggests that it could be implicated in general protein turnover resulting from the intricate interplay of synthetic reactions and degradative processes (HOLZER *et al.*, 1975).

Considering that this protease is most probably located inside the cytoplasm, peptides with no stimulatory properties would be those for which the *bacterium* has no peptide transport system (SUSSMAN and GILVARG, 1971) or periplasmic peptidase.

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RÉSUMÉ

PROPRIÉTÉS GÉNÉRALES ET SPÉCIFICITÉ DE SUBSTRAT D'UNE PROTÉASE INTRACELLULAIRE NEUTRE DE *STREPTOCOCCUS DIACETILACTIS*

La purification, les principales propriétés et la spécificité d'action d'une endopeptidase intracellulaire de *S. diacetilactis* sont décrites dans ce mémoire.

Après broyage des cellules et élimination des acides nucléiques, la protéase était purifiée par chromatographie sur DEAE-cellulose, ECTEOLA-cellulose et Gel-Sephadex G-100. Le poids moléculaire de l'enzyme était estimé à 49 500 daltons par filtration sur Gel-Sephadex G-100. Son activité était maximum à pH 7,0 et à 45°C (avec l'insuline oxydée comme substrat). L'énergie apparente d'activation de la réaction était de 18 000 cal/mole. L'endopeptidase était stable pour les températures inférieures à 37°C. Elle était totalement inactivée par l'EDTA 1mM. Les ions Mn^{++} et Co^{++} la réactivaient complètement après cette inhibition par un chélateur. Le *p*-CMB ne l'inhibait que faiblement mais le Dip-F n'était pas inhibiteur.

Cette protéase hydrolyse rapidement l'insuline oxydée, le glucagon ou la caséine α_{s1} mais ne semble pas attaquer les protéines natives (insuline, ribonucléase, β-lactoglobuline, azocaséine), ni les substrats esters usuels, ni les dipeptides mono- ou disubstitués, ni les dérivés d'acides aminés. La spécificité d'action était déterminée par analyse des peptides des hydrolysats de la chaîne B de l'insuline. Ces peptides étaient isolés sur colonnes de Bio-Rad AG-50 W × 2 et par chromatographie sur papier et électrophorèse sous haute tension. La position des peptides dans la chaîne B de l'insuline et l'estimation de leur concentration montrent que la plupart des liaisons peptidiques et spécialement celles le plus rapidement hydrolysées impliquent le groupe α-aminé d'un résidu hydrophobe (telles les liaisons His_{10}-Leu_{11}, Tyr_{16}-Leu_{17}, Gly_{23}-Phe_{24}). Donc cette endopeptidase intracellulaire appartient au groupe des métallo-protéases neutres (EC. 3.4.24.4).

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