Purification and Characterization of Two Aminopeptidases Produced by *Brevibacterium linens*

By KIYOSHI HAYASHI*† AND BARRY A. LAW

AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT, UK

(Received 28 December 1988; revised 13 March 1989; accepted 22 March 1989)

Two aminopeptidases, designated as aminopeptidase A and B, were purified 1720- and 1950-fold, respectively, from the culture filtrate of *Brevibacterium linens* F by ammonium sulphate fractionation, a series of column chromatography steps on DEAE-Sephadex, DEAE-Trisacryl M and Mono-Q, and gel filtration on Superose-6. Aminopeptidase A accounted for 85% of the aminopeptidase activity remaining by the end of purification. The purified enzymes were homogeneous as judged by disc gel electrophoresis. Aminopeptidases A and B showed the same pH optimum of 9.3 and apparent temperature optimum of 40 °C, although the former showed slightly higher pH stability than the latter. NaCl or KCl concentrations to 2 M did not affect the activities or stabilities of aminopeptidases A and B. Both enzymes were completely inhibited by incubation with EDTA, indicating that they are metalloenzymes, and were reactivated by incubation with Ca²⁺, Co²⁺, Mg²⁺, Zn²⁺ or Mn²⁺. The *M_r* values of aminopeptidases A and B were estimated as 150 000 and 110 000, respectively, by gel filtration on Superose-6, and as 36 000 and 26 000, respectively, by SDS-PAGE. It was thus assumed that each of the native enzymes exists as a tetramer. The *K_m* values of aminopeptidase A and B for L-leucine-*p*-nitroanilide were calculated to be 16.1 and 15.2 mM, respectively. The substrate specificities of aminopeptidases A and B were similar to each other.

INTRODUCTION

Brevibacterium linens is an important component of the surface microflora of smear-ripened cheese varieties (e.g. Danbo, Limburger, Gruyère), where it is involved in the ripening process, in changing the texture of the cheese, and in providing amino acids as substrates for flavour-producing enzymes (e.g. deaminase, demethylase; Law, 1984).

B. linens produces both extracellular proteinase and peptidase activity. The present paper deals largely with the aminopeptidase. Foissy (1978*a, b, c*) purified and characterized a single aminopeptidase produced by *B. linens*. However, multiple forms of peptide hydrolase active on Leu-Tyr have been reported from this species (Sørhaug, 1981; Torgersen & Sørhaug, 1978). For example, Sørhaug (1981) claimed, based on the results of zymograms of crude extracts, that one strain produced five electrophoretically distinct peptide hydrolases active on Leu-Tyr.

In this report, we describe the purification and characterization of two aminopeptidases produced by *B. linens* F. We show that the purified aminopeptidases have a broad specificity which is considered to be appropriate for their role in cheese ripening.

METHODS

Micro-organisms and culture conditions. *Brevibacterium linens* F, which was kindly donated by Chr. Hansen's Laboratory Ltd, Wisconsin, USA, was cultivated aerobically on an orbital incubator at a speed of 250 r.p.m. in a medium composed of tryptone 1% (w/v), glucose 0.5%, soytone 0.3%, yeast extract 0.1%, K₂HPO₄ 0.25% and MgSO₄·7H₂O 0.02%, pH 7.0. After 48 h of cultivation at 24 °C, the cells were harvested by centrifugation at 5000 *g* for 10 min at 4 °C.

† Present address: National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Kannondai, Tsukuba, Ibaraki 305, Japan.

Abbreviations: LNA, L-leucine-*p*-nitroanilide; *p*-NA, *p*-nitroanilide.

Measurement of enzyme activities. Proteinase activity was measured at pH 9.0 using casein as a substrate. The enzyme reaction was carried out at 30 °C for 30 min and then released peptides were measured by the method of Folin & Ciocalteu (1927). Aminopeptidase activity was determined in 50 mM-Tris/HCl buffer at pH 8.5 at 30 °C using L-leucine-*p*-nitroanilide (LNA) (1.74 mM) as a substrate (Foissy, 1978*a*). Methanol (0.1%) was included in the assay mixture (2 ml) owing to the low solubility of LNA. One unit of activity is defined as the amount of enzyme which produces 1 μmol *p*-nitroaniline min^{-1} under the standard condition.

Buffers used to investigate the effects of temperature and pH on aminopeptidase activity. All buffers were used at a concentration of 50 mM. Thermal stability and optimum temperature were measured in Tris/HCl buffer, pH 8.5. Acetate buffer of pH 5.0–5.8, 1,3-bis[tris(hydroxymethyl)methylamino]propane buffer of pH 5.6–10.0 and 3-(cyclohexylamino)-1-propanesulphonate buffer of pH 9.5–11.3 were used for the measurement of pH stability. In order to investigate optimum pH, the following additional buffers were used: phosphate buffer, pH 6.5–8.1; *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonate) buffer, pH 6.8–8.3; Tris/HCl buffer, pH 7.0–9.0; glycine buffer, pH 9.0–11.0.

Purification of aminopeptidase. The separations with Mono-Q and Superose-6 (Pharmacia LKB Biotechnology Co.) were carried out at room temperature and all other purification steps at 4 °C.

Solid ammonium sulphate was added to the culture supernatant (10 litres) to a final saturation of 90%. The precipitate was collected by centrifugation at 10000 *g* for 30 min and dissolved in 110 ml 20 mM-phosphate buffer, pH 7.0. The solution was dialysed overnight against 10 litres of the same buffer containing 0.2 M-NaCl.

Dialysed solution (120 ml) containing 3.15 g protein was applied to a DEAE-Sephadex A-50 (Pharmacia LKB Biotechnology Co.) column (3.2 $\times$ 27 cm) previously equilibrated with the same buffer containing 0.2 M-NaCl. The column was eluted with a linear gradient of NaCl from 0.2 to 0.8 M in the buffer (1300 ml). Fractions (12 ml) were collected and assayed for proteinase and aminopeptidase activity.

Aminopeptidase fractions were pooled and dialysed against 20 mM-Tris/HCl buffer (pH 8.0) containing 0.15 M-NaCl. The dialysed solution was applied to a column (1.6 $\times$ 25 cm) containing DEAE-Trisacryl M (Pharmacia LKB Biotechnology Co.) equilibrated with the same buffer containing 0.15 M-NaCl. The column was eluted with a linear gradient of NaCl from 0.15 to 0.5 M (500 ml) and fractions (6 ml) containing aminopeptidase were pooled and stored at –20 °C.

Part of the fraction (about 0.5 mg protein) was applied to a column of Mono-Q HR5/5 (0.5 $\times$ 5 cm) equilibrated with 25 mM-piperazine/HCl buffer, pH 6.0. The column was eluted with a linear gradient of NaCl in the buffer (40 ml) from 0.3 to 0.5 M. The active fractions were pooled and concentrated by using a Collodion bag (Sartorius).

A portion of pooled concentrates (about 0.5 mg protein) was loaded on a gel filtration column of Superose-6 HR10/30 (1 $\times$ 30 cm) previously equilibrated with 20 mM-Tris/HCl buffer (pH 8.0) containing 0.3 M-NaCl. The active fractions were pooled and used as purified enzyme fractions of aminopeptidase A and B. The last two steps were repeated as required.

Determination of K_m and V_{max} . In order to calculate K_m and V_{max} , enzyme activity was measured twice over different concentrations of substrates; Leu-Leu (from 15 to 100 mM), Leu-Arg (from 2 to 20 mM) and LNA (from 0.85 to 3 mM). The reaction was initiated by adding enzyme (6.8 milliunits in 40 μl) to the preincubated substrate solution of 50 mM-Tris/HCl buffer, pH 8.8 (360 μl). Change of absorbance at 235 nm (for Leu-Leu), 230 nm (for Leu-Arg) or 410 nm (for LNA) was recorded for 5 to 30 min to calculate the initial rate of hydrolysis at 30 °C.

Substrate specificity. The initial rates of hydrolysis of peptides were measured twice at 30 °C in borate buffer (pH 9.0) by the modified ninhydrin method (Doi *et al.*, 1981).

Polyacrylamide gel electrophoresis (PAGE). PAGE for aminopeptidase A (10 μg) and B (7 μg) was performed at pH 8.3 by the method described by Ornstein (1964). Activity staining was performed by laying gels on an agar plate containing 1 mg LNA ml^{-1} and 10 mM-Tris/HCl buffer, pH 8.5. SDS-PAGE for aminopeptidase A (20 μg) and aminopeptidase B (14 μg) was performed in 10% (w/v) polyacrylamide disc gels with the phosphate buffer system described by Weber & Osborn (1969).

Estimation of M_r . M_r values of the purified enzymes were estimated by gel filtration on a Superose-6 HR10/30 column using 20 mM-Tris/HCl buffer, pH 8.0, containing 0.3 M-NaCl. Horse spleen apoferritin (M_r 443000), bovine liver catalase (232,000), sweet potato β -amylase (200000), rabbit muscle aldolase (158000), yeast alcohol dehydrogenase (150000), bovine albumin (66000), hen egg ovalbumin (45000) and whale myoglobin (17200) were used as reference proteins for gel filtration.

Rabbit muscle phosphorylase *b* (M_r 97400), bovine albumin (66000), hen egg ovalbumin (45000), pepsin (34700), chymotrypsinogen (25700), trypsin (23300), whale myoglobin (17200) and horse cytochrome *c* (12400) were used as the standard marker proteins in SDS-PAGE (Weber & Osborn, 1969).

Protein determination. Protein content was determined spectrophotometrically with an assumed absorption coefficient at 280 nm of 10.0 for a 1% (w/v) solution.

Chemicals. All optically active amino acids used were of the L-configuration, unless otherwise stated. All marker proteins, peptides and amino acid derivatives used were purchased from Sigma. Tryptone, soytone and yeast extract were from Difco. All other chemicals were obtained as analytical grade from commercial sources.

RESULTS

Purification of aminopeptidases

Two proteinase peaks and one aminopeptidase peak were eluted on DEAE-Sephadex column chromatography. Separation of proteinase from aminopeptidase activity was achieved by column chromatography on Mono-Q HR5/5. In a final purification step, a small portion of the active fraction was applied to a Superose-6 HR10/30 column and separated further into two peaks. The main peak, which contained 85% of the total activity remaining at the final purification step, was designated as aminopeptidase A and the minor one as aminopeptidase B. Purified enzyme fractions of aminopeptidases A and B were obtained by reapplying each fraction to the gel filtration column, and these were used throughout the following experiments. A summary of the purification procedure is shown in Table 1. The enzyme activities of aminopeptidases A and B were purified 1720- and 1950-fold, respectively, giving specific activities of 53.1 U (mg protein)⁻¹ and 60.2 U (mg protein)⁻¹. The homogeneities of aminopeptidases A and B were determined by PAGE (Fig. 1 A, B). The enzyme activity of the bands of aminopeptidases A and B was confirmed by activity staining.

M_r values

The *M_r* values of aminopeptidases A and B were estimated to be 150 000 and 110 000, respectively, by Superose-6 gel filtration. When aminopeptidase A or B was dissociated in 0.1% SDS containing 1% (v/v) 2-mercaptoethanol, and subjected to electrophoresis, single bands were observed after staining (Fig. 1 C, D). The migration of these bands corresponded to *M_r* values of 36 000 for aminopeptidase A and 26 000 for aminopeptidase B. The two native enzymes were thus each considered to be composed of four identical subunits.

Effect of temperature and pH on the activities and stabilities of the enzymes

Activity and stability at pH 8.5 were measured at different temperatures from 25 to 60 °C. The apparent optimum temperature for activity was 40 °C for both aminopeptidases A and B. The enzymes were both stable below 40 °C when incubated for 15 min. The pH optima for aminopeptidases A and B were nearly the same (pH 9.3) when measured at 30 °C. The two enzymes were inactive on LNA at pH values below 5.5. The effect of pH on the stability of the enzymes at 30 °C was determined by incubation in several buffers for 2 h. The two aminopeptidases were most stable at their optimum pH of 9.3. Aminopeptidase A was slightly more stable than aminopeptidase B at pH values below 8.

Table 1. *Purification of the extracellular aminopeptidases from B. linens*

Purification step	Volume (ml)	Activity (U ml ⁻¹)	Specific activity (U mg ⁻¹)	Yield (%)	Purification (-fold)
Culture filtrate	10000	0.0897	0.0308	100	1
Ammonium sulphate fractionation	120	10.1	0.385	136	12.5
DEAE-Sephadex A-50 chromatography	105	7.47	15.3	87.7	498
DEAE-Trisacryl M chromatography	72	3.97	21.3	32.0	692
Mono-Q HR5/5 chromatography*	—	—	48.1	(24.7)*	1560
Superose-6 HR10/30 gel filtration*†					
Aminopeptidase A	—	—	53.1	(12.0)*	1720
Aminopeptidase B	—	—	60.2	(2.19)*	1950

* Only a portion of sample was applied in these steps as columns are semipreparative size. Yields were calculated based on the application of a portion of sample to the column.

† The aminopeptidase fraction was separated into two fractions, aminopeptidase A and B, in this step.

Fig. 1. Homogeneities of the purified aminopeptidase A (A) and aminopeptidase B (B) on polyacrylamide gel; C, D, aminopeptidase A (C) and aminopeptidase B (D) on SDS-polyacrylamide gel. All gels were stained with Coomassie brilliant blue R-250.

Effect of metal ions and EDTA on the enzymes

None of the metal ions tested enhanced the activities of the two enzymes; they were not affected by Ba^{2+} , Ca^{2+} or Mg^{2+} , but were strongly inhibited by Cu^{2+} , Mn^{2+} , Zn^{2+} , Hg^{2+} and Cd^{2+} (Table 2). Both enzymes completely lost their activity when incubated with 0.1 mM-EDTA. The EDTA-inactivated aminopeptidases A and B were reactivated by incubation with Ca^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} or Mn^{2+} (Table 3).

Analysis of K_m and substrate specificity

The K_m values of aminopeptidases A and B for LNA were 16.1 and 15.2 mM, respectively. The K_m of aminopeptidase A for Leu-Arg and Leu-Leu was 5.25 and 85 mM, respectively.

Table 4 summarizes the initial rates of hydrolysis of peptides and amino acid derivatives by aminopeptidase A. The K_m values obtained for Leu-Arg, LNA and Leu-Leu indicate that the substrate concentrations for these three substrates, and probably for other peptides, are not

Table 2. *Effect of various metal ions on aminopeptidase A and B activities*

Enzyme (17 milliunits of aminopeptidase A or B) was preincubated for 1 h at 30 °C with the metal salt indicated. After incubation, the activity was measured by the standard assay. Enzyme activity in the absence of metal salts measured by the standard assay was defined as 100%.

Metal salt (1 mM)	Relative activity (%)	
	Aminopeptidase A	Aminopeptidase B
BaCl ₂	98.6	99.4
CaCl ₂	96.9	93.3
MgCl ₂	90.4	91.3
CoCl ₂	32.4	32.0
AlCl ₃	21.1	18.1
FeCl ₂	14.8	16.4
CuCl ₂	8.4	7.5
MnCl ₂	5.1	5.8
ZnCl ₂	1.5	1.4
HgCl ₂	1.2	2.1
CdCl ₂	0.6	1.2
SnCl ₂ *	9.7	11.1
NiCl ₂ *	2.1	3.4
PbCl ₂ *	0.6	1.2
None	100	100

* Precipitate was observed during the enzyme reaction.

Table 3. *Reactivation of EDTA-inactivated aminopeptidases A and B by several metal ions*

The native enzyme (17 milliunits of aminopeptidase A or B) was incubated with 0.1 mM-EDTA at 0 °C for 1 h, then metal salt was added to a concentration of 0.1 mM. After incubation for 30 min at 0 °C, the enzyme was assayed. The final concentrations of metal ion and EDTA in the standard assay were 0.04 mM and 0.004 mM, respectively. The activity of the native enzyme which was not treated by EDTA was measured in the absence of metal salts and is defined as 100%.

Metal salt	Extent of reactivation (%)	
	Aminopeptidase A	Aminopeptidase B
CaCl ₂	99.5	98.0
CoCl ₂	89.3	87.1
MgCl ₂	47.0	36.8
ZnCl ₂	36.4	26.7
MnCl ₂	20.5	33.2
None	0	0

saturation. Therefore, the initial rates of hydrolysis of these peptides reflect both K_m and V values. The following substrate specificities for aminopeptidase A can be deduced: the substrates with an N-terminal leucine were the most readily hydrolysed among the tested peptides; a strong specificity was observed with regard to the second amino acid, since in a series of dipeptides with N-terminal leucine the rate of hydrolysis varied by a factor of 324 (relative activity of 3.4 for Leu-Gly and 1100 for Leu-Arg); not only dipeptides but also tripeptides and tetrapeptides were hydrolysed; an amino acid derivative which has an acidic amino acid (Glu-pNA) is not a substrate for this enzyme. Peptides consisting of D-amino acids such as D-Leu-D-Leu were not hydrolysed by either of the two enzymes. Substrate specificities of the two enzymes were very similar to each other.

Table 4. *Substrate specificities of aminopeptidase A*

To minimize experimental error, enzyme concentration (1.5–30 milliunits) and incubation time (5–180 min) were varied depending on the susceptibility of each substrate.

Substrate*	Relative activity†	Substrate*	Relative activity†
Gly-Leu	<0.1	Tyr-Leu	28.8
-Phe	<0.9	Phe-Ala	<2.5
Ala-Ala	3.6	-Leu	4.3
-Pro	3.6	-Phe	7.9
-Val	6.0	-Phe-Phe	49.1
-Leu	5.0	-Phe-Phe-Phe*	21.8
-Phe	11.8	Lys-Ile	2.2
-Lys	11.0	-Phe	5.5
Val-Ala	1.8	His-Ala	2.6
-Pro	<2.9	-Leu	19.8
-Phe	11.2	-Lys	60.0
Met-Gly	0.8	-Phe	25.0
-Pro	<0.4	Arg-Leu	5.5
Leu-NH ₂	26.0	Try-Ala	3.7
-Ser	42.7		
-Pro	3.7	Pro-Gly	0.3
-Gly	3.4	-Met	15.2
-Ala	52.9	-Leu	0.9
-Val	91.8	-Tyr	4.9
-Met	408 (435)	-Phe	4.5
-Ile	42.5	-Try	4.6
-Leu	100 (100)		
-Tyr	268 (216)	Glu-pNA	<0.02
-Phe	187 (182)	Ala-pNA	10.4
-Arg	1100 (1364)	Leu-pNA	745 (764)
-Try	227 (239)	Lys-pNA	39.8
-Leu-Leu	131		
D-Leu-D-Leu	<0.1 (<0.1)		
D-Leu-L-Leu	<0.1 (<0.1)		
L-Leu-D-Leu	<0.1 (<0.1)		

* All amino acids were in the L-configuration, except where indicated. Concentration of substrate was 1 mM except for tetraphenylalanine (about 0.4 mM).

† Rate of hydrolysis relative to that of Leu-Leu, for which 6.7% of the substrate was hydrolysed after incubation with aminopeptidase A or B (3 milliunits) at 30 °C for 10 min. Figures in parentheses are for aminopeptidase B.

DISCUSSION

The aminopeptidases investigated in this study were purified from the culture filtrate of *B. linens* F. A previous study of peptide hydrolase of the same species by a zymogram technique suggested the presence of more than one enzyme (Sørhaug, 1981; Torgersen & Sørhaug, 1978). This has been confirmed by our isolation of aminopeptidases A and B; the former showed 85% of the total activity remaining at the final purification step.

The physical and chemical properties of aminopeptidases A and B were very similar. The two enzymes showed the same apparent temperature optimum of 40 °C and the same pH optimum of 9.3, although a slight difference was found in pH stability, aminopeptidase A being slightly more stable at acidic pH than aminopeptidase B. K_m values for LNA hydrolysis by the two enzymes were similar: 16.1 mM for aminopeptidase A and 15.2 mM for aminopeptidase B. The two enzymes are considered to be metalloenzymes since they were inactivated by treatment with EDTA and reactivated by incubation with various divalent metal ions. Evidence that each enzyme consists of four identical subunits was obtained from SDS-PAGE. In contrast, Foissy (1978*b*) reported the aminopeptidase of *B. linens* to be a dimer consisting of identical monomers of M_r 48000. The only prominent difference found between aminopeptidases A and B was in their estimated M_r , 150000 for native aminopeptidase A and 110000 for aminopeptidase B.

The two enzymes were so similar in their properties that they may be regarded as isoenzymes of group 1 (genetically independent proteins) or group 4b (conjugated or derived proteins, proteins derived from single polypeptide chains) (IUPAC-IUB CBN, 1977). Multiple forms of peptidases have also been reported from other organisms (Hasegawa *et al.*, 1985; Kim & Brophy, 1976; Rabier & Desmazeaud, 1973). It is possible that the difference in M_r between aminopeptidases A and B is due to different carbohydrate contents, as reported for peptidases F and S from rat brush border membrane peptidase (Kim & Brophy, 1976). It is also possible that aminopeptidase A was converted to aminopeptidase B by specific proteolytic hydrolysis, although the incubation of aminopeptidase A with proteinase isolated from the same strain failed to produce aminopeptidase B. Until further information is available the term 'multiple forms of the enzyme' should be used.

The effect of Co^{2+} on the activity of aminopeptidases A and B is not entirely consistent with that on other similar metalloenzymes. Activation of native enzyme by incubation with Co^{2+} was observed in the enzyme purified from *B. linens* (Foissy, 1978a), *Streptococcus cremoris* (Hwang *et al.*, 1981), *Streptococcus faecalis* (White & Clewell, 1986) and *Bacillus stearothermophilus* (Zuber & Roncari, 1967). More than 100% of the original activity was observed with enzyme renatured by incubation with Co^{2+} for the enzyme purified from *Thermus aquaticus* (Minagawa *et al.*, 1988) and *Escherichia coli* (Yoshimoto *et al.*, 1988). However, we observed slight inhibition of native enzyme and only partial renaturation of EDTA-denatured enzyme by incubation with Co^{2+} for aminopeptidases A and B.

The purified aminopeptidases A and B hydrolysed a variety of substrates, showing a specificity for N-terminal leucine. Their substrate specificity differed from that of the aminopeptidase purified by Foissy (1978b). A marked difference was observed in the ratio of the reaction velocity for L-leucinamide and LNA: aminopeptidase A had a ratio of 29:1 with the respective substrates, while the enzyme reported by Foissy (1978b) had a ratio of 1:6. The specificity of the purified aminopeptidase A was not restricted to dipeptides; tripeptide and tetrapeptide substrates were also hydrolysed. Proline iminopeptidase activity (Yoshimoto & Tsuru, 1985) was also confirmed, as the enzyme hydrolysed L-prolyl-L-methionine and related substrates. This broad substrate specificity of the enzyme, which hydrolyses peptides containing N-terminal hydrophobic, aromatic and basic amino acids as well as proline, seems ideal for the purpose of accelerated ripening of cheese (Law & Wigmore, 1983).

The aminopeptidase I of *E. coli* is sensitive to the monovalent ion concentration (Vogt, 1970), but the enzyme activities and stabilities of aminopeptidases A and B measured in 50 mM-Tris/HCl buffer pH 8.5 were not affected by NaCl or KCl concentration up to 2 M. No metal ion was required for the enzyme activity of the aminopeptidases isolated from *B. linens* F while the presence of Mn^{2+} and Co^{2+} was essential for the activity of aminopeptidase of *Clostridium histolyticum* (EC 3.4.11.13, Kessler & Yaron, 1973). From the data described here, aminopeptidases A and B appear to belong to the same classification as *Aeromonas proteolytica* aminopeptidase [α -aminoacyl-peptide hydrolase (*Aeromonas proteolytica*), EC 3.4.11.10] (IUB, 1984), although some differences between the aminopeptidases isolated from *A. proteolytica* and *B. linens* F were observed in the substrate specificity for L-leucinamide and LNA, and in the rate of reactivation by metal ions (Prescott & Wilkes, 1966; Wilkes *et al.*, 1973).

This work was supported by Japan International Cooperation Agency. We thank Mr A. J. Cliff (AFRC Institute of Food Research, Reading Laboratory) for his kind support of the experiments, Dr C. H. Tzeng (Chr. Hansen's Laboratory Co.) for kindly providing the strain and Dr A. Hosono (Shinshuu University) for useful discussion.

REFERENCES

- DOI, E., SHIBATA, D. & MATOBA, T. (1981). Modified colorimetric ninhydrin methods for peptidase assay. *Analytical Biochemistry* **118**, 173–184.
- FOISSY, H. (1978a). Aminopeptidase from *Brevibacterium linens*. *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **166**, 164–166.
- FOISSY, H. (1978b). Some properties of aminopeptidase from *Brevibacterium linens*. *FEMS Microbiology Letters* **3**, 207–210.
- FOISSY, H. (1978c). Aminopeptidase from *Brevibacterium linens*, production and purification. *Milchwissenschaft* **33**, 221–223.

- FOLIN, O. & CIOCALTEU, V. (1927). On tyrosine and tryptophane determinations in proteins. *Journal of Biological Chemistry* **73**, 627–650.
- HASEGAWA, Y., KODAMA, O. & AKATSUKA, T. (1985). Solubilization and some properties of membrane-bound aminopeptidases of hog small intestinal mucosa. *Nippon Nogeikagaku Kaishi* **59**, 397–403.
- HWANG, I. K., KAMINOGAWA, S. & YAMAUCHI, K. (1981). Purification and properties of a dipeptidase from *Streptococcus cremoris*. *Agricultural and Biological Chemistry* **45**, 159–165.
- IUB (1984). *Enzyme Nomenclature 1984*. Orlando: Academic Press.
- IUPAC-IUB CBN (1977). Nomenclature of multiple forms of enzymes, recommendations (1976). *Journal of Biological Chemistry* **252**, 5939–5941.
- KESSLER, E. & YARON, A. (1973). A novel aminopeptidase from *Clostridium histolyticum*. *Biochemical and Biophysical Research Communications* **50**, 405–412.
- KIM, Y. S. & BROPHY, E. J. (1976). Rat intestinal brush border membrane peptidases. *Journal of Biological Chemistry* **251**, 3199–3211.
- LAW, B. A. (1984). Flavour development in cheeses. In *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*, pp. 187–208. Edited by F. L. Davies & B. A. Law. London: Elsevier Applied Science Publishers.
- LAW, B. A. & WIGMORE, A. S. (1983). Accelerated ripening of cheddar cheese with a commercial proteinase and intracellular enzymes from starter streptococci. *Journal of Dairy Research* **50**, 519–525.
- MINAGAWA, E., KAMINOGAWA, S., MATSUZAWA, H., OHTA, T. & YAMAUCHI, K. (1988). Isolation and characterization of a thermostable aminopeptidase from *Thermus aquaticus* YT-1. *Agricultural and Biological Chemistry* **52**, 1755–1763.
- ORNSTEIN, L. (1964). Disk electrophoresis. 1. Background and theory. *Annals of the New York Academy of Sciences* **121**, 321–349.
- PRESCOTT, J. M. & WILKES, S. H. (1966). *Aeromonas* aminopeptidase, purification and some general properties. *Archives of Biochemistry and Biophysics* **117**, 328–336.
- RABIER, D. & DESMAZEAUD, M. J. (1973). Inventory of different intracellular peptidase activities of *Streptococcus thermophilus*. *Biochimie* **55**, 389–404.
- SØRHAUG, T. (1981). Comparison of peptide hydrolases from six strains of *Brevibacterium linens*. *Milchwissenschaft* **36**, 137–139.
- TORGERSEN, H. & SØRHAUG, T. (1978). Peptide hydrolases of *Brevibacterium linens*. *FEMS Microbiology Letters* **4**, 151–153.
- VOGT, V. M. (1970). Purification and properties of an aminopeptidase from *Escherichia coli*. *Journal of Biological Chemistry* **245**, 4760–4769.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.
- WHITE, B. A. & CLEWELL, D. B. (1986). Identification and partial purification of a dipeptidyl aminopeptidase from *Streptococcus faecalis*. *Journal of General Microbiology* **132**, 1269–1276.
- WILKES, S. H., BAYLISS, M. E. & PRESCOTT, J. M. (1973). Specificity of *Aeromonas* aminopeptidase toward oligopeptides and polypeptides. *European Journal of Biochemistry* **34**, 459–466.
- YOSHIMOTO, T. & TSURU, D. (1985). Proline iminopeptidase from *Bacillus coagulans*. *Journal of Biochemistry* **97**, 1477–1485.
- YOSHIMOTO, T., TAMESA, Y., GUSHI, K., MURAYAMA, N. & TSURU, D. (1988). An aminopeptidase N from *Escherichia coli* HB101. *Agricultural and Biological Chemistry* **52**, 217–225.
- ZUBER, H. & RONCARI, G. (1967). Thermophilic and mesophilic aminopeptidases from *Bacillus stearothermophilus*. *Angewandte Chemie-International English Edition* **6**, 880–881.