ELSEVIER

Food Chemistry 65 (1999) 15–22

Food Chemistry

Rapid communication

Purification and characterization of an acid phosphatase from *Lactobacillus plantarum* DPC2739

Abdallah A.A. Magboul*, Paul L.H. McSweeney

Department of Food Science and Technology, University College, Cork, Ireland

Received 17 September 1998; accepted 14 October 1998

Abstract

An acid phosphatase was partially purified from a cell-free extract of *Lactobacillus plantarum* DPC2739 by a combination of anion-exchange chromatography on DEAE-Sephacel, hydrophobic interaction chromatography on Phenyl Sepharose, gel permeation chromatography on Sephacryl S200 and high performance anion-exchange chromatography on MonoQ. The native enzyme (~110 kDa) was tetrameric with a subunit molecular mass of ~27 kDa. The enzyme was heat-stable, retaining ~60% of its activity after heating for 30 min at 70°C. It was optimally active in the pH range 3.5–5.0 and at 40°C. The enzyme was strongly inhibited by 0.5 mM sodium fluoride, and hexametaphosphate and by 5 mM orthophosphate, tripolyphosphate and pyrophosphate. It was insensitive to metal chelators (ethylenediaminetetraacetic acid and *o*-phenanthroline), ascorbic acid, sulphydryl blocking agents (e.g., N-ethylmaleimide), phenylmethylsulphonyl fluoride and divalent metal ions at 5 mM concentration. The enzyme appeared to be a non-specific phosphomonoesterase and hydrolysed a number of phosphate esters. The amino acid sequence of the first 20 residues was determined and showed some homology with mammalian, yeast and *Escherichia coli* acid phosphatases, phosphoglycerate mutases and phosphoglycerokinases with a common motif Arg-His-Gly.

1. Introduction

Phosphatases are phosphomonoesterases which catalyse the hydrolysis of the C–O–P linkage of a wide variety of phosphate esters. They are classified as acid or alkaline phosphatases depending on their pH optima (Stauffer, 1989). These enzymes are apparently ubiquitous in nature, occurring in many animal tissues, plants and microorganisms (Waymack & Van Etten, 1991). Although both acid and alkaline phosphatases are present in cheese, the former are more active due to their relatively low optimum pH (~5.0). During cheese ripening, phosphate-rich peptides are produced which appear to be resistant to further proteolysis (Fox, Law, McSweeney, & Wallace, 1993) due to the protective effect of the phosphate residues. The combined action of acid phosphatases and the proteolytic system in cheese is thus required for extensive production of small peptides and free amino acids (Larsen & Parada, 1988). Several phosphopeptides, originating from α_{s1}−, α_{s2}− or β-caseins, were identified in Cheddar cheese by Singh, Fox, and Healy (1997) and these authors observed extensive dephosphorylation of some peptides. Although acid phosphatase activity is present in cheese, the origin of these enzymes is controversial (Fox et al. 1993). Possible origins of acid phosphatases in cheese are from bovine milk and/or from the cheese microflora (Larsen & Parada, 1988). Bovine milk contains a heat-stable acid phosphatase which is capable of dephosphorylating phosphoproteins, including the caseins. The purification of this enzyme was reported by Bingham and Zittle (1963). Acid phosphatase activity was detected in whole cells and crude enzyme extracts from *Lactococcus lactis* subsp. *cremoris* Wg2 and *Lactobacillus delbrueckii* subsp. *bulgaricus* ACAD235; the enzyme activity of the *Lactococcus* strain was 10-fold higher than that of the *Lactobacillus* (Kyriakidis, Sakellaris, & Sotiroudis, 1993).

Lactobacillus plantarum is a component of the non-starter lactic acid bacteria (NSLAB) which dominate the microflora of mature Cheddar and other cheeses (McSweeney, Fox, Lucey, Jordan, & Cogan, 1993). Acid phosphatases from NSLAB might be of importance in the dephosphorylation of phosphopeptides produced during cheese ripening since the lactococcal phosphatase does not dephosphorylate casein to a significant extent (Fox et al., 1993). The aim of this study was to purify and characterize an acid phosphatase

* Corresponding author. E-mail: dydk6014@bureau.ucc.ie

0308-8146/99/$—see front matter
PII: S0308-8146(98)00255-6

from *Lactobacillus plantarum* DPC2739 and study its specificity on different substrates including casein.

2. Materials and methods

2.1. Reagents

Diethylaminoethyl (DEAE)-Sephacel, Phenyl Sepharose and Sephacryl S200 were obtained from Pharmacia LKB Biotechnology Inc., Uppsala, Sweden. Phosphate esters, malachite green and molecular weight standards for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography were obtained from Sigma Chemical Co., St Louis, MO, USA. Deoxyribonuclease (DNase) I and ribonuclease (RNase) were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents used were of analytical grade.

2.2. Microorganism, growth conditions and preparation of cell-free extracts

Lb. plantarum DPC2739 was obtained from the Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. It was pre-cultivated in de Man, Rogosa and Sharpe (MRS) broth (Unipath Ltd., Basingstoke, Hampshire, England) and cultivated in 20 litres of the same medium (1% inoculum) overnight (~16 h) at 30°C. The culture was harvested by centrifugation at 5000 ***g*** for 15 min at 4°C and cells were washed twice with 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 7.0. The recovered cells were used to prepare a cell-free extract.

The washed cells were resuspended in 50 mM Tris-HCl buffer, pH 7.0, and disrupted by sonication for 15 min using a Soniprep 150 MSE (Sanyo, Leeds, UK) in 15 s blasts at 22 μA, with pauses of 30 s. During sonication, the temperature was maintained at ~0°C with a mixture of ice and ethanol. Intact cells and cell debris were removed by centrifugation at 47 000 ***g*** for 15 min at 4°C. The supernatant was used as crude extract for purification.

2.3. Enzyme assay

Acid phosphatase activity was determined by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (pNPP). The assay mixture contained 0.5 ml of 12 mM pNPP in 0.2 M sodium acetate buffer, pH 5.2, and 0.5 ml of enzyme solution was added. The mixture was incubated at 40°C for 60 min and the reaction was terminated by the addition of 1.0 ml of 1.0 M NaOH. The *p*-nitrophenol released was determined spectrophotometrically by measuring its absorbance at 405 nm. One enzyme unit was defined as the amount of enzyme catalysing the liberation of 1.0 μ mol of *p*-nitrophenol per min under the conditions of the assay. In the case of other substrates, the quantity of inorganic phosphate released from phosphate esters was measured by the method of Geladopoulos, Sotiroudis, and Evangelopoulos (1991) and the activity was expressed as μ mol inorganic phosphate released per min under the assay conditions described above.

2.4. Enzyme purification

All chromatographic steps were performed at ~20°C. The protein contents of the column effluents were determined by measuring their absorbances at 280 nm. The Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) was used according to the manufacturer's instructions to measure the protein content of the cell-free extract and pooled fractions. Bovine serum albumin was used as a standard.

The cell-free extract was concentrated by ultrafiltration (UF) using a Minitan ultrafiltration unit fitted with polysulphone membranes with 10 kDa nominal molecular weight cutoff (Millipore Corp., Bedford, MA, USA). The retentate was dialysed against 50 mM Tris-HCl buffer, pH 7.0, for 6 h at 4°C. The concentrated sample was applied to a DEAE-Sephacel column (20×1.6 cm) and proteins were eluted with a linear NaCl gradient (0–0.35 M) in 50 mM sodium acetate buffer, pH 5.6, at a flow rate of 48 ml h^{-1}. The flow rate, salt gradient and fraction size were controlled using a Gradifrac system (Pharmacia). Active fractions were pooled and concentrated by centrifugation at 3000 ***g*** for 3 h using Centriprep concentrating cartridges (Amicon Beverly, MA 01915, USA) with 10 kDa nominal molecular weight cutoff.

The concentrated active fraction from DEAE-Sephacel was applied to a Phenyl Sepharose column (40×1.0 cm), equilibrated with 20 mM sodium acetate buffer, pH 5.6 containing 1 M $(NH_4)_2SO_4$. The proteins were eluted using a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia) at a flow rate of 30 ml h^{-1} with a gradient from 1 to 0 M $(NH_4)_2SO_4$ in 20 mM sodium acetate buffer, pH 5.6. Active fractions were pooled and concentrated as described above.

The concentrated active fraction from hydrophobic interaction chromatography was then applied to a Sephacryl S200 column (70×1.6 cm) and eluted using a Gradifrac system (Pharmacia) with 20 mM acetate buffer, pH 5.5 containing 0.1 M NaCl at a flow rate of 30 ml h^{-1}. The active fractions were combined, concentrated as described above, dialysed for 6 h at 4°C against 20 mM acetate buffer, pH 5.5, and stored at ~0°C.

High performance (FPLC) ion-exchange chromatography on MonoQ HR 5/5 (Pharmacia) was used to further purify the concentrated active fraction from

chromatography on Sephacryl S200. Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM acetate buffer, pH 5.5, and the active fractions were pooled and stored at ~0°C for subsequent characterization.

2.5. Thermal stability, temperature and pH optima

To check the thermal stability of the enzyme, equal volumes of enzyme solution in 20 mM sodium acetate buffer, pH 5.2, were pre-incubated for 10–30 min at 50, 60 or 70°C and the residual activity was assayed at 40°C and expressed as a percentage of the activity of an unheated sample. For determination of the temperature optimum, enzymatic activity was measured in 20 mM sodium acetate buffer, pH 5.2, at various temperatures from 20 to 60°C. Acid phosphatase activity was determined at 0.5 pH unit intervals between pH 2.0 and 9.0 at 40°C in 0.1 M glycine (pH 2.0–3.0), sodium acetate (pH 3.5–6.5) or Tris-HCl (pH 7.0–9.0) buffers. The activity at each pH value was expressed as a percentage of the maximum activity.

2.6. Effect of inhibitors and metal ions

The enzyme solution was dialysed for 6 h at 4°C against water prior to incubation at ~20°C for 30 min with inhibitors or metal ions at a concentration of 0.5 or 5 mM. Residual activity was measured at pH 5.2 and 40°C using pNPP as substrate. The relative activity was calculated as the percentage of an untreated control.

2.7. Determination of enzyme purity and molecular mass

The cell-free extract and pooled active fractions after each chromatographic step were examined by SDS-PAGE as described by Laemmli (1970). The molecular mass of the native acid phosphatase was estimated by gel permeation chromatography on a TSK G2000 SW column fitted with a TSK SW guard column (Toso-HAAS, Cambridge, UK) using a Shimadzu high performance liquid chromatography (HPLC) system (consisting of a DG-2410 degassing unit, SPD-6A UV spectrophotometric detector, LC-9A Pump and SIL-GA auto-injector; Shimadzu Corp., Kyoto, Japan). The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl and 0.05% (w/v) sodium azide. For molecular mass determination, the column was calibrated with proteins of known molecular weights (MW-GF-200 Kit, Sigma). The molecular weight of the enzyme was also determined under denaturing conditions by SDS-PAGE. Electrophoresis was performed under denaturing (SDS) and non-denaturing conditions in 12% (w/v) acrylamide gels and proteins were visualized by staining with Coomassie Brilliant Blue R-250 (0.1% w/v). The non-denaturing gel was cut into two parts, one of which was stained and the other was cut into slices corresponding to the protein bands; acid phosphatase activity was detected by incubation of the slices at 40°C in 0.2 M acetate buffer, pH 5.2, containing 6 mM pNPP.

2.8. Substrate specificity

Inorganic phosphate released from a number of phosphate esters (1 mM concentration) incubated with the partially purified acid phosphatase at pH 5.2 and 40°C was determined as described above.

2.9. N-Terminal amino acid sequence

Proteins were electroblotted from both denaturing (SDS) and non-denaturing PAGE onto polyvinylidene difluoride (PVDF) membranes (Applied Biosystems, Foster City, CA, USA) according to a method described by Singh, Fox and Healy (1995). The stained protein band representing the acid phosphatase was cut from the blot and sequenced for 20 cycles from its N-terminus using a pulsed liquid phase Procise 494 protein sequencer, with a 785A programmable absorbance detector and interfaced with a Macintosh Quadra 650 computer (Applied Biosystems Inc.). For amino acid homology and sequence alignment, the EMBL database was searched using the Beauty CRS Blast sequence database search tool (Worley, Wiese, & Smith, 1995).

3. Results

3.1. Enzyme purification

An acid phosphatase from *Lb. plantarum* DPC2739 was partially purified using a four-step chromatographic procedure. In the first step on DEAE-Sephacel, two active peaks were detected [Fig. 1(a)]. The first peak was not retained on the DEAE-Sephacel and eluted in the void volume. The second peak, which contained most of the acid phosphatase activity, eluted at ~0.18 M NaCl and was studied. This step resulted in about 7-fold increase in specific activity over the concentrated cell-free supernatant and an activity yield of 62.4% (Table 1). In the second purification step on Phenyl Sepharose [Fig. 1(b)], the acid phosphatase activity was eluted at ~0.2 M $(NH_4)_2SO_4$. This chromatographic step gave ~18-fold increase in specific activity with a yield of 26.7% over the concentrated cell-free extract (Table 1). The third chromatographic step on Sephacryl S200 [Fig. 1(c)] yielded 20% of the original acid phosphatase activity (Table 1) representing an approximately 41-fold purification. In the final purification step on MonoQ [Fig. 1(d)], a 62-fold increase in specific activity over the concentrated cell-free supernatant was obtained with a final yield of 6.2% (Table 1). The

cell-free extract and the pooled active fractions after each chromatographic step were analysed by SDS-PAGE [Fig. 2(A)] and the band corresponding to the acid phosphatase activity determined under non-denaturing conditions is shown in Fig. 2(B). After the final chromatographic step on MonoQ, two protein bands were detected by SDS-PAGE [Fig. 2(A)] with molecular weights between 24 and 29 kDa. On excision from the native gel, only the protein band with the greater molecular weight had acid phosphatase activity [Fig. 2(B)].

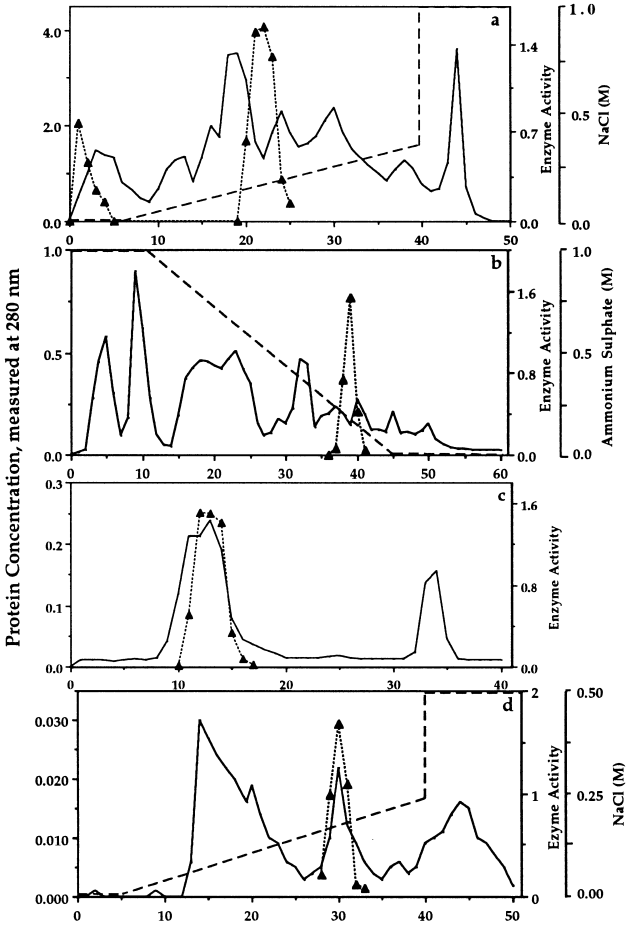

Fig. 1. Purification of the acid phosphatase of *Lb. plantarum* DPC2739. Elution profiles obtained after (a) ion-exchange chromatography on DEAE-Sephacel, (b) hydrophobic interaction chromatography on Phenyl Sepharose, (c) gel permeation chromatography on Sephacryl S200 and (d) high performance ion-exchange chromatography on MonoQ. Protein (——A_{280}), acid phosphatase activity (···▲···A_{405}) and salt (– – –).

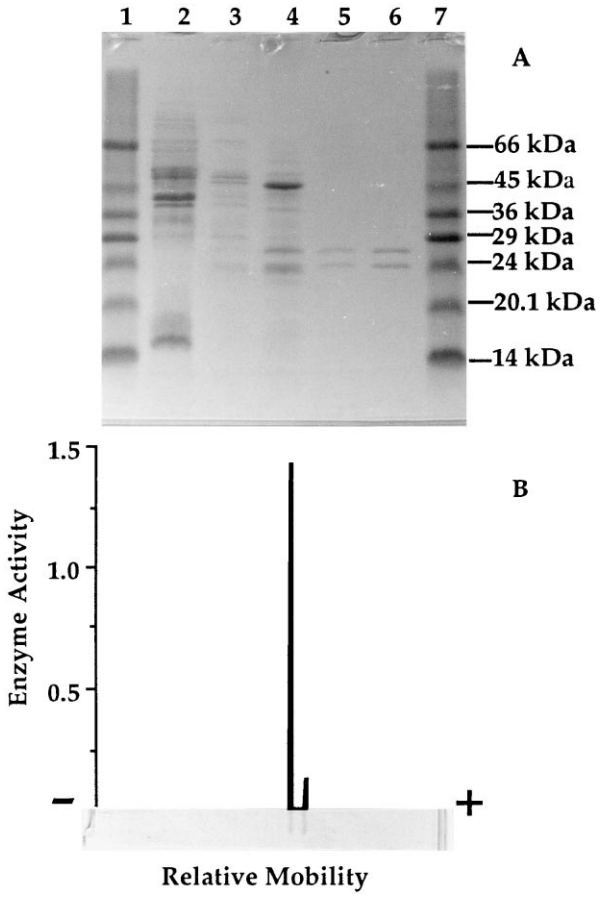

Fig. 2. (A) SDS-PAGE of the cell-free extracts of *Lb.plantarum* DPC2739 (lane 2) and pooled active fractions after chromatography on DEAE-Sephacel (lane 3); Phenyl Sepharose (lane 4); Sephacryl S200 (lane 5) and MonoQ HR 5/5 (lane 6). Lanes 1 and 7 are molecular weight markers. (B) Electrophoresis under non-denaturing conditions of the acid phosphatase active fraction after MonoQ and the enzyme activity of the corresponding region of the gel excised before staining.

Table 1
Purification of an acid phosphatase from *Lb. plantarum* DPC2739

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Fold increase in specific activity	Activity yield (%)
CCFE[a]	19 868	13 710	6.9	1	100
DEAE-Sephace1[b]	244	8556	35.1	7	62.4
HIC[c]	29	3654	126	18	26.7
S200[d]	10	2819	281.9	41	20.6
MonoQ[e]	2	850	425	62	6.2

[a] Concentrated cell-fee extract.
[b] Anion-exchange chromatography on DEAE-Sephacel.
[c] Hydrophobic interaction chromatography on Phenyl Sepharose.
[d] Gel permeation chromatography on Sephacryl S200.
[e] High performance anion-exchange chromatography on MonoQ 5/5HR.

3.2. Determination of molecular mass

The apparent molecular mass for the acid phosphatase from *Lb. plantarum* DPC2739 was estimated to be ~27 kDa by SDS-PAGE [Fig. 2(a)] and ~110 kDa by gel permeation chromatography (data not shown).

3.3. Effect of temperature and pH

The thermal stability of the enzyme in 20 mM sodium acetate buffer, pH 5.2, was determined at 50, 60 and 70°C after 10, 20 and 30 min incubation. The acid phosphatase retained ~90%, of its initial activity after pre-incubation for 30 min at 50°C, ~75% activity at 60°C and ~60% activity at 70°C (Fig. 3).

Acid phosphatase activity in 20 mM sodium acetate buffer, pH 5.2, was optimum at 40°C and with greater than 60% of its maximum activity at 35, 50 and 55°C (Fig. 4).

The acid phosphatase exhibited optimum activity in the pH range 3.5–5.5 (>80% relative activity) with an optimum activity at pH 4.0 (Fig. 5).

3.4. Effect of inhibitors and metal ions

The effect of inhibitors and metal ions on acid phosphatase activity is shown in Table 2. The enzyme was strongly inhibited by 0.5 mM NaF and hexametaphosphate and by 5 mM orthophosphate, tripolyphosphate and pyrophosphate. On the other hand, ethylenediaminetetraacetic acid phenylmethylsulphonyl fluoride, N-ethylmaleimide and ascorbic acid, had no or little effect on acid phosphatase activity (Table 2).

The enzyme activity was unaffected by the divalent metal ions tested (Table 2) at 5 mM concentration, since no significant inhibition or stimulation of activity were observed.

3.5. Substrate specificity

Activity of the partially purified acid phosphatase on various phosphate esters at pH 5.2 and 40°C is summarized in Table 3. The acid phosphatase exhibited high activity on pNPP, D-glucose-6-phosphate, *o*-phospho-DL-serine, D-fructose-6-phosphate, but only low activity on *o*-phospho-DL-threonine, *o*-phospho-DL-tyrosine, pyrophosphate, α-D-glucose-l-phosphate and uridine-5′-monophosphate. The enzyme had very little or no activity on adenosine-5′-monophosphate, β-glycerophosphate, α-naphthyl phosphate, adenosine-5′-di-phosphate, β-naphthyl phosphate, D-glucose-1,6-di-phosphate,

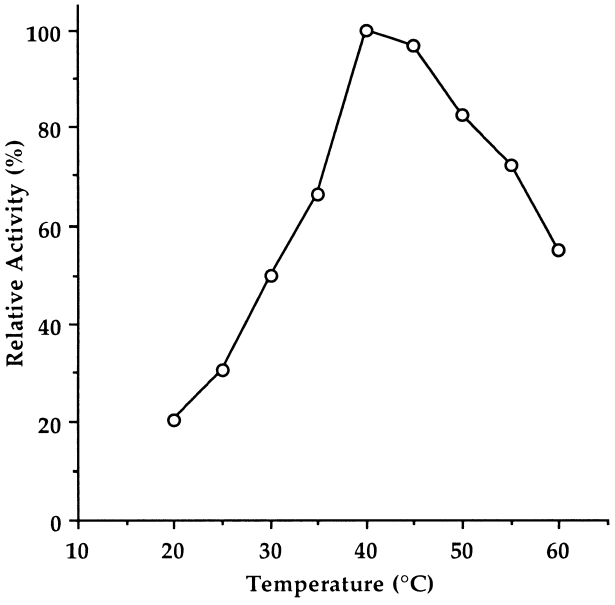

Fig. 4. Effect of temperature on the activity of the acid phosphatase from *Lb. plantarum* DPC2739 at pH 5.2.

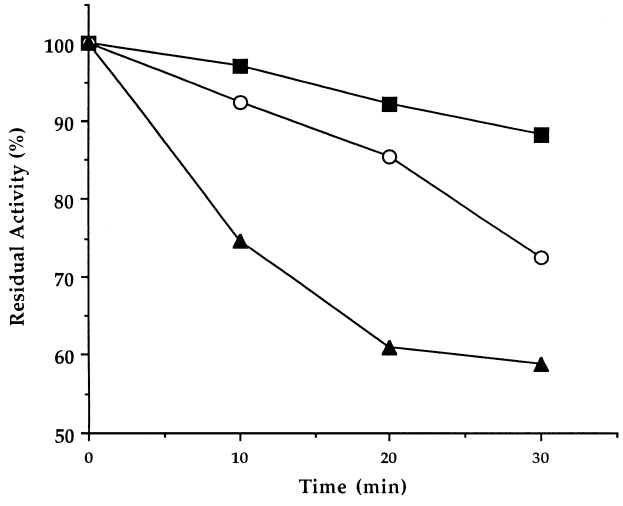

Fig. 3. Thermal stability of the acid phosphatase from *Lb. plantarum* DPC2739 determined at 50°C (■), 60°C (○) and 70°C (▲) in 20 mM Na–acetate buffer, pH 5.2.

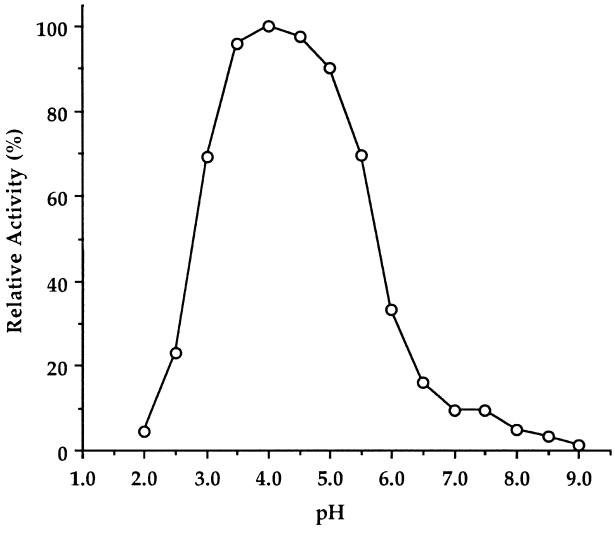

Fig. 5. Effect of pH on the activity of the acid phosphatase from *Lb. plantarum* DPC2739 at 40°C.

Table 2
Effect of inhibitors and metal ions on the activity of the acid phosphatase from *Lb. Plantarum* DPC2739

Compound	Concentration (mM)	Residual activity (%)
None	0	100
Ethylenediaminetetraacetic acid	5	95
o-Phenanthroline	5	100
Phenylmethylsulphonyl fluoride	5	96
Sodium fluoride	0.5	0.5
N-Ethylmaleimide	5	97
Ascorbic acid	5	105
Hexametaphosphate	0.5	5
β-Glycerophosphate	5	82
Pyrophosphate	5	24
Tripolyphosphate	5	7
Orthophosphate	5	0
$BaCl_2$	5	101
$CaCl_2$	5	102
$CoCl_2$	5	96
$CuCl_2$	5	101
MgO_2	5	100
$MnCl_2$	5	102
$ZnCl_2$	5	100

Table 3
Specificity of the acid phosphatase from *Lb. plantarum* DPC2739 on different substrates

Substrate	μ mol inorganic phosphate released[a]
p-Nitrophenyl phosphate	92
α-Naphthyl phosphate	3
β-Naphthyl phosphate	0
D-Fructose-6-phosphate	48
α-D-Glucose-1-phosphate	16
D-Glucose-6-phosphate	77
D-Fructose-1,6-phosphate	0
Adenosine-5′-monophosphate	8
Adenosine-5′diphosphate	2
Adenosine-5′-triphosphate	0
Uridine-5′-phosphate	12
bis-(*p*-Nitrophenyl) phosphate	0
β-Glycerophosphate	3
Pyrophosphate	20
Tripolyphosphate	0
Sodium caseinate[b]	1.2
o-Phospho-DL-serine	51
o-Phospho-DL-threonine	34
o-Phospho-DL-tyrosine	25

[a] Inorganic phosphate released after 60 min incubation at 40°C.
[b] Na-caseinate (1 mg ml^{-1}) was incubated with enzyme for 17 h before assaying for the released inorganic phosphate.

adenosine-5′-triphosphate, bis (*p*-nitrophenyl) phosphate and tripolyphosphate. Incubation of sodium caseinate (1 mg $litre^{-1}$) with the partially purified enzyme (50 μl) for 17 h at 40°C resulted in the release of 1.2 μ mol of inorganic phosphate.

3.6. N-Terminal amino acid sequencing

The N-terminal amino acid sequence for the first 20 amino acid residues was determined as H_2N-AQF-SIYFVRHGQTFFNLYNR, for the band of lower electrophoretic mobility under both denaturing [Fig. 2(a)] and non-denaturing conditions [Fig. 2(b)]. The band with higher mobility under denaturing conditions [Fig. 2(a)] had an N-terminal amino acid sequence of H_2N-ATFSVYMIRHGQTYFNKYRR.

Amino acid sequence alignments for the acid phosphatase from *Lb. plantarum* DPC2739 and fructose-6-phosphate-2-kinases/fructose-2, 6-biphosphatases, phosphoglycerate mutases and acid phosphatases from different species are shown in Table 4.

4. Discussion

The purification procedure used in this study resulted in a fraction with acid phosphatase activity containing two proteins as determined by gel electrophoresis under denaturing (SDS) and non-denaturing conditions [Fig. 2(a),(b)]. The acid phosphatase activity in this fraction coincided only with the band of lower electrophoretic mobility under non-denaturing conditions [Fig. 2(b)]. The sequence of the first 20 amino acid residues was identical for the lower mobility band from both native and denaturing gels, suggesting that the proteins migrated in the same order on both gels. The protein with greater electrophoretic mobility was excised from the native gel and assayed for acid phosphatase activity. However this protein had no more activity than could be accounted for by diffusion [Fig. 2(b)], strongly suggesting that there is only one active acid phosphatase in the final preparation from the cell-free extract of *Lb. plantarum* DPC2739. The higher mobility band showed significant sequence homology (65%) with the lower mobility band; both bands had a similar molecular mass (as determined by SDS-PAGE, Fig. 2a) and co-eluted on gel permeation chromatography on TSK G2000 SW. These results may explain the difficulty encountered in separating these two proteins by chromatography. To our knowledge, acid phosphatase activities of lactic acid bacteria have only been characterized in crude, or partially purified, preparations (Andrews & Alichanidis, 1975a; Kyriakidis et al., 1993). There appears to be no report on purification to homogeneity of an acid phosphatase from lactic acid bacteria.

Molecular mass determination by SDS-PAGE (~27 kDa) and gel permeation chromatography (~110 kDa) suggested that the native enzyme exists as a tetramer with identical subunits. Bovine milk acid phosphatase consists of a single glycosylated polypeptide chain of ~42 kDa while no molecular masses have been reported

Table 4
Alignments of sequences of the acid phosphatase (AP) from *Lactobacillus curvatus* DPC2024[a] and fructose-6phosphatate-2-kinase/fructose-2, 6-biphosphatases (kinase), phosphoglycerate mutases (mustase) and acid phosphatases from different species. Amino acid sequence were obtained from the EMBL data base[b] or from Ostanin et al. (1992)[c]

Amino acid sequence																				Type of enzyme	Species
A_1	**Q**	**F**	**S**	**I**	**Y**	**F**	**V**	**R**	**H**	**G**	**Q**	**T**	**F**	**F**	**N**	**L**	**Y**	**N**	**R_{20}**	AP	*Lb. curvatus*[a]
T_{249}	P	R	A	**I**	**Y**	L	S	**R**	**H**	**G**	E	S	Q	L	**N**	**L**	K	G	**R_{268}**	Kinase	Chicken[b]
H_{249}	P	R	T	**I**	**Y**	L	C	**R**	**H**	**G**	E	S	E	**F**	**N**	**L**	L	G	**K_{268}**	Kinase	Rat[b]
T_{219}	P	R	**S**	**I**	**Y**	L	S	**R**	**H**	**G**	E	S	E	L	**N**	**L**	L	G	**R_{238}**	Kinase	Frog[b]
T_{250}	P	R	**S**	**I**	**Y**	L	C	**R**	**H**	**G**	E	S	E	L	**N**	I	R	G	**R_{270}**	Kinase	Human[b]
A_2	A	Y	K	L	V	L	I	**R**	**H**	**G**	E	S	A	W	**N**	**L**	E	N	**R_{21}**	Mutase	Rat[b]
	M_1	P	T	L	V	L	S	**R**	**H**	**G**	**Q**	S	E	W	**N**	**L**	E	N	**R_{19}**	Mutase	*Z. mobilis*[b]
A_2	T	H	R	L	V	M	**V**	**R**	**H**	**G**	E	**T**	T	W	**N**	Q	E	**N**	**R_{21}**	Mutase	Human[b]
A_2	V	T	K	L	V	L	**V**	**R**	**H**	**G**	E	S	Q	W	**N**	K	E	**N**	**R_{21}**	Mutase	*E. coli*[b]
S_2	K	Y	K	L	I	M	L	**R**	**H**	**G**	E	G	A	W	**N**	K	E	**N**	**R_{21}**	Mutase	Rabbit[b]
L_3	E	S	V	V	I	V	S	**R**	**H**	**G**	V	R	A	P	T	K	A	T	Q_{22}	AP	*E. coli*[c]
L_3	K	**F**	V	T	L	V	F	**R**	**H**	**G**	D	R	S	P	I	D	T	F	P_{22}	AP	Human prostate[c]
L_3	R	**F**	V	T	L	L	Y	**R**	**H**	**G**	D	R	S	P	V	K	T	Y	P_{22}	AP	Human lysosome[c]
I_3	K	Q	V	H	T	L	Q	**R**	**H**	**G**	S	R	N	P	T	G	G	**N**	A_{22}	AP	Yeast[c]
M_3	K	Q	L	Q	M	V	G	**R**	**H**	**G**	E	R	Y	P	T	V	S	L	A_{22}	AP_5	Yeast[c]
M_3	K	Q	L	Q	M	L	A	**R**	**H**	**G**	E	R	Y	P	T	Y	S	K	G_{22}	AP_3	Yeast[c]

for acid phosphatases from LAB other than the observation that they appear to have high molecular masses (Andrews & Alichanidis, 1975a; Andrews & Alichanidis, 1975b; Kyriakidis et al., 1993).

The enzyme was maximally active in the pH range 3.5–5.0 with optimum activity at pH 4.0 and 40°C. A similar pH optimum was reported for the milk acid phosphatase while acid phosphatases from other LAB showed maximum activity in the pH range 5.0–6.0 (Andrews & Alichanidis, 1975a,b; Larsen & Parada, 1988; Kyriakidis et al., 1993).

Characterization of the acid phosphatase from *Lb. plantarum* DPC2739 was performed at pH 5.2 because of the role that this enzyme might play on the dephosphorylation of phosphopeptides in Cheddar cheese since *Lb. plantarum* is a component of the NSLAB that dominate the microflora of Cheddar cheese during ripening (McSweeney et al., 1993).

The enzyme characterized in this study was strongly inhibited by NaF, hexametaphosphate, polyphosphate and orthophosphate as were phosphatases described previously from milk and LAB (Fox et al., 1993; Kyriakidis et al., 1993). Ascorbic acid, which activates milk acid phosphatate, had no effect on the enzyme characterized in this study, which is in agreement with the effect of ascorbic acid on lactococcal acid phosphatases (Fox et al., 1993). The enzyme was unaffected by N-ethylmaleimide (NEM) and metal ions. The lactococcal enzymes, unlike the milk acid phosphatase, were inhibited by sulphyldryl reagents such as NEM (Fox et al., 1993).

The acid phosphatase from *Lb. plantarum* DPC2739 is a rather non-specific enzyme since it hydrolyses a range of different phosphate esters, (e.g., pNPP, D-glucose-6-phosphate, D-fructose-6-phosphate, pyrophosphate, *o*-phospho-DL-serine, *o*-phospho-DL-threonine, *o*-phospho-DL-tyrosine and Na-caseinate), regardless of the chemical nature of the leaving group esterifying the phosphoryl residue. The enzyme was inactive on bis (*p*-nitrophenyl) phosphate. These results indicate that the enzyme characterized in this study is a typical phosphomonoesterase.

The N-terminal amino acid sequence of the first 20 residues of the acid phosphatase from *Lb. plantarum* DPC2739 showed 15–45% homology with acid phosphatases, fructose-6-phosphate-2-kinases/2-fructose-2, 6-bisphosphatases and phosphoglycerate mutases from different species (Table 4). The motif RHG was a feature in common between the acid phosphatase characterized in this study and the above enzymes. This observation was also reported by Ostanin et al. (1992) for acid phosphatases from *Escherichia coli*, human prostate, human lysosome, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. These authors found that the replacement of R and H residues, localized in a conserved N-terminal RHG motif resulted in the complete elimination of *E. coli* acid phosphatase activity.

Biochemical characterization of acid phosphatases of NSLAB and the study of their action on phosphopeptides is a prerequisite to understand their full contribution to the biochemistry of cheese ripening and flavour development. In this study, an acid phosphatase was partially purified and characterized from *Lb. plantarum* DPC2739 and its activity on different substrates, including casein, was studied. Further study on the specificity and kinetics of this enzyme on phosphopeptides of different chain lengths under conditions similar to those found in cheese is required, since whole casein was a poor substrate for this enzyme as was indicated by a low release of inorganic phosphate.

Acknowledgements

This research was part-funded by grant aid under the Food Sub-Programme of the Operational Programme for Industrial Development which is administered by the Department of Agriculture, Food and Forestry, and supported by National and EU Funds. Our thanks are also extended to Áine Healy for N-terminal amino acid sequencing.

References

Andrews, A. T., & Alichanidis, E. C. (1975a). Acid phosphatase activity in cheese and starters. *Journal of Dairy Research, 42*, 327–339.

Andrews, A. T., & Alichanidis, E. C. (1975b). The acid phosphatases of bovine leucocytes, plasma and the milk of healthy and mastitic cows. *Journal of Dairy Research, 42*, 391–400.

Bingham, E. W., & Zittle, C. A. (1963). Purification and properties of acid phosphatase in bovine milk. *Archives of Biochemistry and Biophysics, 401*, 471–477.

Fox, P. F., Law, J., McSweeney, P. L. H., & Wallace, J. (1993). Biochemistry of cheese ripening. In P. F. Fox (Ed.), *Cheese chemistry, physics and microbiology,* (Vol. 1, pp. 389–438). London: Chapman and Hall.

Geladopoulos, T. P., Sotiroudis, T. G., & Evangelopoulos, A. E. (1991). A malachite green colourimetric assay for protein phosphatase activity. *Analytical Biochemistry, 192*, 112–116.

Kyriakidis, S. M., Sakellaris, G., & Sotiroudis, T. G. (1993). Is protein phosphorylation a control mechanism for the degradation of caseins by lactic acid bacteria? The detection of an extracellular acid phosphatase activity. *Letters in Applied Microbiology, 16*, 295–298.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London), 227*, 196–204.

Larsen, R. F., & Parada, J. L. (1988). Acid phosphatases in some cheeses and starters. *Sciences des Aliments, 8*, 285–294.

McSweeney, P. L. H., Fox, P. F., Lucey, J. A., Jordan, K. N., & Cogan, T. M. (1993). Contribution of indigenous microflora to the maturation of Cheddar cheese. *International Dairy Journal, 3*, 613–634.

Ostanin, K., Harms, E. H., Stevis, P. E., Kuciel, R., Zhou, M.-M., & Van Etten, R.L. (1992). Overexpression, site-directed mutagenisis, and mechanism of *Escherichia coli* acid phosphatase. *Journal of Biological Chemistry, 267*, 22830–22836.

Singh, T. K., Fox, P. F., & Healy, A. (1995). Water soluble peptides in Cheddar cheese: isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. *Journal of Dairy Research, 62*, 629–640.

Singh, T. K., Fox, P. F., & Healy, A. (1997). Isolation and identification of further peptides in diafiltration retentate of the water-soluble fraction of Cheddar cheese. *Journal of Dairy Research, 64*, 433–443.

Stauffer, C. E. (1989). *Enzyme assays for food scientists* (2nd ed.), New York: Van Nostrand Reinhold.

Waymack, P. P., & Van Etten, R. L. (1991). Isolation and characterization of a homogeneous isoenzyme of wheat germ acid phosphatase. *Archives of Biochemistry and Biophysics, 288*, 621–633.

Worley, K. C., Wiese, B. A., & Smith, R. F. (1995). Beauty: an enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. *Genome Research, 5*, 173–184.