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THE PRIMARY STRUCTURE OF PHOSPHOFRUCTOKINASE FROM *LACTOCOCCUS LACTIS*

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Summary: The primary amino acid sequence of phosphofructokinase (EC2.7.1.11) from *Lactococcus lactis*, obtained by Edman analysis of peptides obtained from proteolytic digestions, is
MKRIAVLTSGGDAPGMNAAIRAVVRKAISEGIEVYGINHGYAGMVAGDIF
PLTSASVGDKIGRGGTFLYSARYPEFAQVEGQLAGIEQLKKFGIEGVVVI
GGDGSYHGAMRLTEHGFPAVGLPGTIDNDIVGTDFTIGFDTAVSTVVDAL
DKIRDTSSSHNRTFVVEVMGRNAGDIALNAGIAAGADDISIPELEFKFEN
VVNNINKGYEKGKNHHIIIVAEGVMTGEEFATKLKEAGYKGDLRVSVLGH
IQRGGSPTARDRVLASRMGARAVELLRDGIGGVAVGIRNEELVESPILGT
AEEGALFSLTTEGGIKVNNPHKAGLELYRLNSALNNLNL.

Lactococcus lactis belongs to a group of bacteria which is responsible for the conversion of lactose in milk to lactic acid, thus producing the acid conditions necessary for the formation of cheese. Lactose is taken up by the phosphoenolpyruvate dependent transferase pathway (1) and transported into the bacteria as lactose phosphate, which is hydrolysed to glucose and galactose 6-phosphate. These hexoses are then metabolized to lactic acid by two different pathways. The glucose is converted via the glycolytic pathway whereas the galactose 6-phosphate is converted via the tagatose pathway (2). Since neither galactose 6-phosphate nor glucose 6-phosphate accumulate intracellularly, both the glycolytic pathway and the tagatose pathway must be closely controlled. *Lactococcus lactis* phosphofructokinase (LIPFK), which catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate in the glycolytic pathway has been shown to exhibit allosteric properties similar to those of the PFKs from *Bacillus stearothermophilus* (BsPFK) and *Escherichia coli* (EcPFK) (3). The crystal structures of BsPFK and EcPFK have been described (4,5) and

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the primary structure of PFK from *Thermus thermophilus* (TtPFK) has been deduced from nucleic acid sequences (6). The primary structure of LlPFK is described in this paper for comparison.

Materials and Methods

Growth of bacteria. *Lactococcus lactis* C10 were grown in 35litre batches in a Fermacell Fermentor (New Brunswick Scientific Co) using the medium and growth conditions described by Fordyce (3). Cells were harvested after 6 hours growth, washed with distilled water and stored at -25° until required.

Enzyme purification. *Lactococcus lactis* (100g frozen cells) were suspended in 200ml Tris/glycerol buffer (50mM Tris/HCl pH 7.5 containing 20% glycerol, 5mM $MgCl_2$, 5mM EDTA and 10mM 2-mercaptoethanol) and were disrupted by two passages through a French pressure cell (American Instrument Co) at 38 MPa. The cellular debris and unbroken cells were removed by centrifugation at 27000 x g for 15 minutes. The precipitate was washed once with Tris/glycerol buffer and the initial supernatant and washings were combined. All centrifugations and subsequent purification steps were carried out at 0-4°. The supernatant was applied to a DEAE cellulose column (Whatman DE32, 5x20cm) equilibrated in Tris/glycerol buffer and the PFK was eluted with a gradient of KCl from 0 to 400mM in the Tris/glycerol buffer in a total volume of 2 litres. The fractions containing PFK were collected, pooled and loaded directly onto a Cibacron Blue Sepharose column (2x8cm) prepared by the method of Atkinson (7) and equilibrated with Tris/glycerol buffer. PFK was eluted with 100ml of a linear gradient from 0-2.5M KCl in Tris/glycerol buffer. The fractions containing PFK were pooled, dialysed and applied to a second Cibacron Blue Sepharose column (1.5x5cm) and eluted with a gradient of 0-20mM ATP in Tris/glycerol buffer and stored until required at -20° in Tris/glycerol buffer with the concentration of glycerol raised to 50%. A typical procedure yielded 12mg enzyme of specific activity 160, representing a recovery of 45% of the activity in the initial lysate.

Enzyme assays. PFK was assayed according to the method described by Fordyce (3) and protein concentrations were measured by the method of Lowry (8).

Enzyme digestions. PFK was denatured in 6M guanidine hydrochloride and then freed from denaturing agent by thorough dialysis against distilled water. During the dialysis the PFK precipitated and was collected by centrifugation. 20mg of the precipitated PFK was suspended in 0.1M ammonium bicarbonate and digested with 0.2mg trypsin for 4hr at 37°. The suspension cleared during digestion and after freeze-drying the peptides were fractionated by HPLC on a Vydak 250x4.6mm RPC18 column using a linear gradient from 0.1M ammonium bicarbonate to 0.1M ammonium

bicarbonate:acetonitrile:isopropanol (1:1:1v/v). The fractions obtained were dried and refractionated by HPLC using a linear gradient from 0.1% trifluoroacetic acid in water to 0.08% trifluoracetic acid in 70% acetonitrile. The eluate was monitored continuously at 220nm and fractions were collected manually. Digestions with *Staphylococcus aureus* protease (endoGlu-C) and chymotrypsin using 20mg and 8mg PFK respectively were carried out in a similar manner except that only the trifluoroacetic acid solvent system was used.

Amino acid and sequence analyses. Proteins or peptides were hydrolysed in evacuated, sealed glass tubes with glass distilled 6M HCl containing 0.2% phenol and the amino acids were analysed using an LKB alpha plus analyser. The protein did not contain either cysteine or tryptophan. Sequencing was carried out with an Applied Biosystems model 470 sequencer with a coupled HPLC for PTH analysis.

Mass spectrometry. The peptides which had been isolated by HPLC were analysed by LSIMS mass spectrometry in a glycerol or glycerol/thioglycerol matrix using a VG70-250S double sector mass spectrometer fitted with a cesium ion gun.

Nomenclature of peptides. Peptides were numbered according to their final alignment in the amino acid sequence. T,C and V stand for peptides obtained from digestion with trypsin, chymotrypsin and endoGlu-C respectively. The residue numbering in figure 2 and in the discussion is based on the sequence of BsPFK.

Results and Discussion

The sequence of the first 42 residues of *Lactococcus lactis* PFK was obtained by direct sequencing and the sequence of the remainder of the protein was obtained from separate digestions using trypsin, chymotrypsin and endoGlu-C. The compositions of the peptides were confirmed by their amino acid analyses and in most cases, by the determination of their molecular weights by mass spectrometry. The amino acid sequence of the protein obtained from the digestions is shown in figure 1. The tryptic peptides were sequenced completely except for T3 and T8.

An unusual chymotrypsin-like cleavage occurred at Tyr41 during the trypsin digestion enabling peptides T3a and T3b to be isolated and sequenced to give the full sequence of T3. Tryptic peptide T8 was also too long to be completely sequenced. A brief subdigestion of T8 with chymotrypsin gave the peptides T8C1 and T8C2 and thus the sequence of T8. Acidification of the endoGlu-C digestion with trifluoroacetic acid (final concentration 0.1%) prior to fractionation by HPLC, caused a precipitate to form which was separated, redissolved in 100% formic acid, sequenced and shown to be peptide

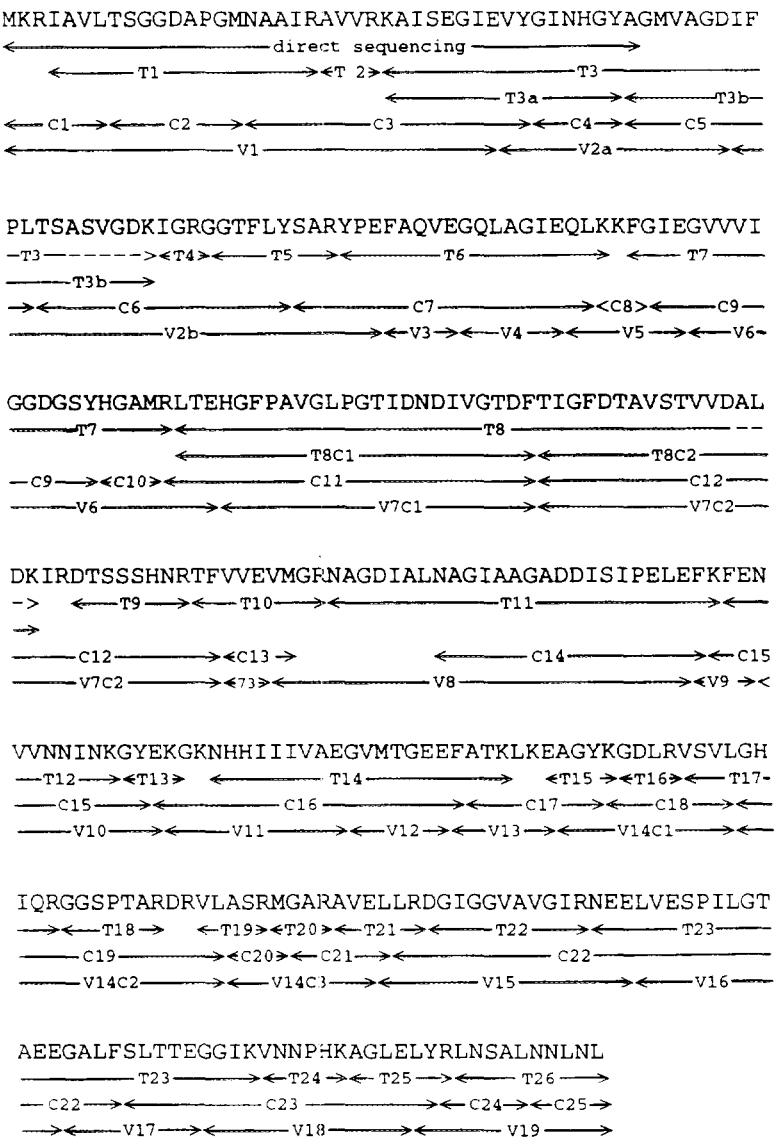

Figure 1. The sequence of *Lactococcus lactis* PFK. Tryptic peptides are labelled T, chymotryptic peptides are labelled C and endoGlu-C peptides are labelled V. Peptides with two letters are from subdigestions. The solid lines indicate that the amino acids were identified by Edman degradation.

V14. The acid soluble peptides were fractionated by HPLC using the trifluoroacetic acid system. Peptides V1-V6,V8-V13 and V15-V19 were sequenced completely. Amino acid analysis confirmed that the Glu-Gly bond in V1 was not cleaved and together with molecular weight determinations by mass spectrometry, confirmed that the cleavage at the Glu-Glu sequences in peptides V12, V15 and V16

occurred after the second glutamic acid residue. There was considerable cleavage at the aspartic acid residue at position 48 leading to the peptides V2a and V2b. The analyses of these two peptides confirmed the sequence of V2. Peptides V7 and V14 were too large to sequence directly. 39 residues of V7 were sequenced directly and the remaining sequence was found by isolating and sequencing the three peptides obtained from a chymotryptic digest of V7 (labelled V7C1,V7C2 and 73 in figure 1). 29 residues of V14 were sequenced directly and the remaining sequence was found in a brief subdigestion with chymotrypsin (V14C1 - V14C3 in figure 1). The information from the chymotryptic digestion of the whole protein was used to obtain overlaps of the peptides produced from trypsin and endoGlu-C and no amino acid analyses or molecular weight analyses were carried out. The sequences of the chymotryptic peptides are shown in figure 1. Peptides V19, T26 and C25 had the C-terminal sequence Asn-Leu-Asn-Leu. Since leucine is not a normal cleavage point for trypsin or endoGlu-C, it was concluded that this sequence was the C-terminus of PFK.

Combination of the data from the three digests enabled the complete sequence to be aligned. Additional evidence for the alignment is gained from a comparison of this sequence with the sequences of EcPFK, BsPFK and TtPFK, which are shown in figure 2. The secondary, tertiary and quaternary structures of BsPFK and EcPFK have been analysed by X-ray crystallography and both enzymes have similar secondary structures including 13 α-helices and 11 β-sheets (9,5). By comparing the sequence of LlPFK with BsPFK and EcPFK and by predicting the secondary structure of LlPFK according to the Chou-Fasman method (10) it is apparent that the secondary structure of LlPFK is quite similar. In most secondary elements, the amino acid residues are similar in all four bacterial PFKs shown in figure 2. Although some residues are different in the different PFKs they have similar abilities to form α–helices, β-pleated sheets or turns and thus the secondary structures are probably not disrupted in any of these PFKs. Many residue changes occur in the helices α-8 (residues 197-212), α-9 (residues 226-239), α-12 (residues 295-302) and α-13 (residues 308-319). In the case of α-8 and α-9 the changes in primary sequence do not prevent the possibility of α-helix formation. It is possible that α-12 of LlPFK is longer than those of Bs or EcPFKs. The residues Gly-Ala-Leu-Phe can be accommodated by increasing the helical portion of α-12 by one turn. The following segment, Ser-Leu-Thr-Thr, would act as a

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     1                                                  50
Bs   M KRIGVLTSGGDSPGMNAAIRSVVRKAIYHGVEVYGVYHGYAGLIAGNIK
Ec   MIKKIGVLTSGGDAPGMNAAIRGVVRSALTEGLEVMGIYDGYLGLYEDRMV
Tt   M KRIGVFTSGGDAPGMNAAIRAVVRQAHALGVEVIGIRRGYAGMIQGEMV
Ll   M KRIAVLTSGGDAPGMNAAIRAVVRKAISEGIEVYGINHGYAGMVAGDIF

     51                                                 100
Bs   KLEVGDVGDIIHRGGTILYTARCPEFKTEEGQKKGIEQLKKHGIEGLVVI
Ec   QLDRYSVSDMINRGGTFLGSARFPEFRDENIRAVAIENLKKRGIDALVVI
Tt   PLGVRDVANIIQRGGTILLTARSQEFLTEEGRAKAYAKLQAAGIEGLVAI
Ll   PLTSASVGDKIGRGGTFLYSARYPEFAQVEGQLAGIEQLKKFGIEGVVVI

     101                                                150
Bs   GGDGSYQGAK KLTEHGFPCVGVPGTIDNDIPGTDFTIGFDTALNTVIDAI
Ec   GGDGSYMGAM RLTEMGFPCIGLPGTIDNDIKGTDYTIGFFTALSTVVEAI
Tt   GGDGTFRGALFLVEEHGMPVVGVPGTIDNDLYGTDYTIGFDTAVNTALEAI
Ll   GGDGSYHGAM RLTEHGFPAVGLPGTIDNDIVGTDFTIGFDTAVSTVVDAL

     151                                                200
Bs   DKIRDTATSHERTYVIEVMGRHAGDIALWSGLAGGAETILIPEADYDMND
Ec   DRLRDTSSSHQRISVVEVMGRYCGDLTLAAAIAGGCEFVVVPEVEFSRED
Tt   DRIRDTAASHERVFFIEVMGRHAGFIALDVGLAGGAEVIAVPEEPVDPKA
Ll   DKIRDTSSSHNRTFVVEVMGRNAGDIALNAGIAAGADDISIPELEFKFEN

     201                                                250
Bs   VIARLKRGHERGKKHSIIIVAEGV GSGVD FGRQIQEATGFETRVTVLGHV
Ec   LVNEIKAGIAKGKKHAIVAITEH  MCDVDELAHFIEKETGRETRATVLGHI
Tt   VAEVLEASQRRGKKSSIVVVAEGAYPGGAAGLLAAIREHLQVEARVTVLGHI
Ll   VVNNINKGYEKGKNHHIIIVAEGV MTGEEFATKLKEAGYKGDLRVSVLGHI

     251                                                300
Bs   QRGGSPTAFDRVLASRLGARAVELLLEGKGGRCVGIQNNQLVDH DIAEAL
Ec   QRGGSPVPYDRILASRMGAYAIDLLLAGYGGRCVGIQNEQLVHH DIIDAI
Tt   QRGGSPTAKDRILASRLGAPAVEALVGGASGVMVGEV EGEVDLTPLKEAV
Ll   QRGGSPTARDRVLASRMGARAVELLRDGIGGVAVGIRNEELVESPILGTAE

     301
Bs   ANKHTIDQRMYALSKELSI
Ec   ENMKRPFKGDWLDCAKKLY
Tt   ERRKDINRALLRLSQVLAL
Ll   EGALFSLTTEGGIKVNNPHKAGLELYRLNSALNNLNL
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Figure 2. Sequences of *Bacillus stearothermophilus* PFK(11), *Escherichia coli* PFK(12) and *Thermus thermophilus* (6) are tabulated for comparison with *Lactococcus lactis* PFK. The numbering of the amino acid residues is based on that of *Bacillus stearothermophilus* PFK. Spaces are included in the sequences to permit the best matching of the proteins.

helix breaking sequence. The remaining extra residues are helix forming and could extend the helix α-13. α-8, α-9, α-12 and α-13 are at the outside loops of the enzyme and are far away from the active site and none of them is involved at the interface of the subunits (5). Thus the amino acid changes in these helices should not affect the association of the subunits and the function of the enzyme. Helix α-8 and the carboxyl terminus of the enzyme are close to the effector binding site and the amino acid alterations in these places could affect the allosteric control characteristics of the enzyme.

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