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# Chloroplast ribosomal protein L15, like L1, L13 and L21, is significantly larger than its *E. coli* homologue

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The purification and identification by peptide sequence and immunological data of the spinach chloroplast homologue of *E. coli* L15 is presented. A significant increase in its mass over the *E. coli* counterpart is shown and is accounted for, in part, by a sequenced 18-residue N-terminal extension. A still larger C-terminal extension or internal insertion(s) is inferred. The migration position of the L15 in a 2D gel pattern of spinach chloroplast 50S subunit proteins is shown. Lack of sequence identity with the known chloroplast genomic data confirms the nuclear coding of this protein, and the N-terminal sequence given here provides the transit peptide cleavage site of the cytoplasmic precursor.

Chloroplast ribosome; Ribosomal protein L15; Chloroplast-bacterial immunohomology; Spinach; *Spinacia oleracea*

## 1. INTRODUCTION

The nuclear-coded chloroplast ribosomal proteins (r-proteins) of higher plants which have been analyzed at the cDNA level maintain a number of unusual characteristics: specifically, N- and C-terminal extensions that increase their mass over that of the *Escherichia coli* homologues, e.g. L13, L21 [1,2]; plastid specific proteins with no homologues on the *E. coli* ribosome [3–5]; and the presence of certain regulatory elements in the transit peptide coding region [6]. For these reasons, characterization of additional nuclear-coded chloroplast r-proteins has assumed a special significance.

Previous data [7,8] combined with the chloroplast genome sequence [9] have now shown that 6 nuclear-coded proteins of the spinach chloroplast 50S ribosomal subunit, namely L1, L3, L6, L12, L13 and L17 are immunological homologues to *E. coli* r-proteins. We have now extended this work to include L15 which gave inconclusive results with the antisera available previously [8]. Here we describe the purification of spinach chloroplast L15 protein and the immunological and peptide sequence homology between it and the *E. coli* and *Bacillus stearothermophilus* L15s. The chloroplast L15 is significantly larger than its *E. coli* and *Bacillus* counterparts.

The sequence and immunology data show the presence of a significant N-terminal extension and a still larger C-terminal extension (or internal addition) in the chloroplast L15. The N-terminal sequence of the purified protein establishes the transit peptide cleavage site in the cytoplasmic precursor.

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*Abbreviations:* r-protein, ribosomal protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline (10 mM $NaH_2PO_4$, pH 7.2, 150 mM NaCl); L-proteins, large ribosomal subunit proteins

## 2. MATERIALS AND METHODS

Spinach (*Spinacia oleracea* cv Alwaro) chloroplast r-proteins reported in this paper are derived from a pool (No. 45) of prefractionated r-proteins described previously [10].

### 2.1. *Protein and peptide purification, protein digestion and sequencing*

The proteins in the pool were purified by RP-HPLC on a 300 Å, 5 μm Vydac C4 column, using water pumps [501(A)/510(B)] and a gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid. The Lys-C protease digestion, peptide purification and automated amino acid sequencing were according to ref. [3].

### 2.2. *Raising of antisera, SDS-PAGE, Western blotting immunostaining and 2D gel electrophoresis*

Antisera were raised (sheep) as described [11]. The *B. stearothermophilus* TP50 proteins and L15 antiserum (from RP-HPLC-purified L15 protein) were provided by Volker Kruft (Research Group Wittmann-Liebold). SDS-PAGE, 2D-PAGE and Western blotting were done as previously described [3,12,13]. For immunostaining [3,12], the antisera were diluted 1:2500 in PBS and reacted at room temperature for 3 h with the Western blotted Immobilon (Millipore) membrane and then for 1.5 h with a secondary antiserum (peroxidase-conjugated rabbit anti-sheep IgG, Dianova GmbH). Bound antibodies were revealed by the developer 0.5 mg/ml 4-chloro-1-naphthol/0.03% $H_2O_2$ in PBS.


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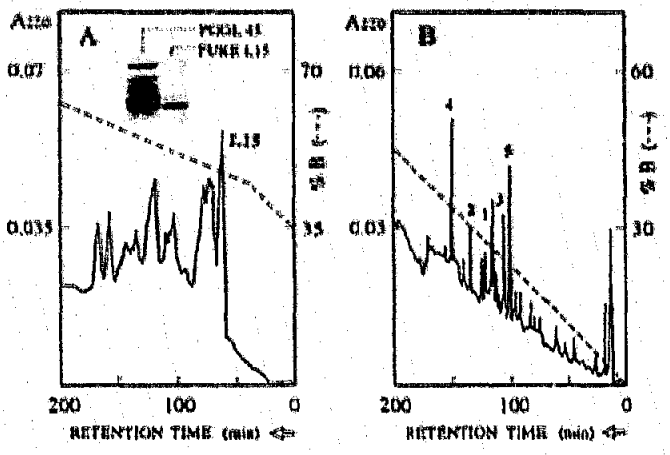


Fig. 1. RP-HPLC profile of pool 45 proteins [10] from which spinach chloroplast L15 was purified. (A) Purity is shown by SDS-PAGE (inset) and the L15 peak is indicated. (B) The purification chromatogram of a Lys-C endoprotease digest of L15. Peptide peaks analyzed on the sequencer are indicated. Broken line in A and B: the gradient of acetonitrile.

| | |
|---|---|
| L15 | SASSSNVSPSIGSGSETRP |
| Peptide 1 | SASSSNVSPSIGSGSETRFRLDNLGPQPG |
| Peptide 2 | SRSGPGIMRGFEGGQMPLYR??P |
| Peptide 3 | GIINPSGRERRLPLK |
| Peptide 4 | YVPINLRDIEVAGFK |
| Peptide 5 | NLARADEYFAK |

Fig. 2. The N-terminal amino acid sequence of spinach chloroplast L15 protein (top) and of 5 peptides from the Lys-C endoprotease digest of L15.

## 3. RESULTS

### 3.1. *Protein/peptide purification and sequencing*

Fig. 1A shows the elution profile, gradient conditions, and SDS-gel electrophoresis results for the purification of spinach chloroplast L15. The chloroplast L15 protein eluted as the first peak from the C4 column.

The protein, determined to be pure by SDS-PAGE (Fig. 1A), shows a molecular mass (measured against marker proteins) of 22 kDa. The N-terminal sequence determined by automated sequencing is shown in Fig. 2. In computer comparison with all the known protein sequences in the NBRF/Swiss Protein/RIBO (this

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SolcL15  SASSSNVSPSIGSGSETRFRLDNLGPQPG                          39
EcoL15                    MRLNTLSPAEGSKKAGKRLGRGIGSGLGKTGGRGHKGQK
BstL15                    MKLHELQPAPGSRKKAVRVGRGIGSGNGRTSGRGQKGQN
                          * *  * ** ** *   * ******* * * *** ***

SolcL15  SRSGPGIMRGFEGGQMPLYRXXP                    80
EcoL15   SRSGGGVRRGFEGGQMPLYRRLPKFGFTSRKAAITAEIRLSDLAKVEGGV-VDLNTL
BstL15   ARSGGGVRLGFEGGQTPLFRRLPKRGFTNINRKEYAVVNLEKLNRFEDGTEVTPELL
          ******* ****** ** ***** ***       *   *   *   * *  *    *

SolcL15     GIINPSGRERRLPLK      120      YVPINLRDIEVAGFK
EcoL15   KAANIIGIQIEFAKVILAGEVTTPVTVRGLRVTKGARAAIEAAGGKIEE   (144)
BstL15   LETGVISKLKSGVKILGKGQIEKKLTVKAHKFSASAKEAIEAAGGKTEVI  (146)
            *       *   *      **          *  ********  *
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Fig. 3. Alignment of spinach L15 peptide sequences (Solc) with *E. coli* (Eco) and *B. stearothermophilus* (Bst) L15 sequences. Stars below the alignment indicate identical residues between the bacterial L15 proteins. Identities between the spinach and *E. coli* proteins are boxed. Data: spinach, this paper; *E. coli* [14]; *B. stearothermophilus* [16].

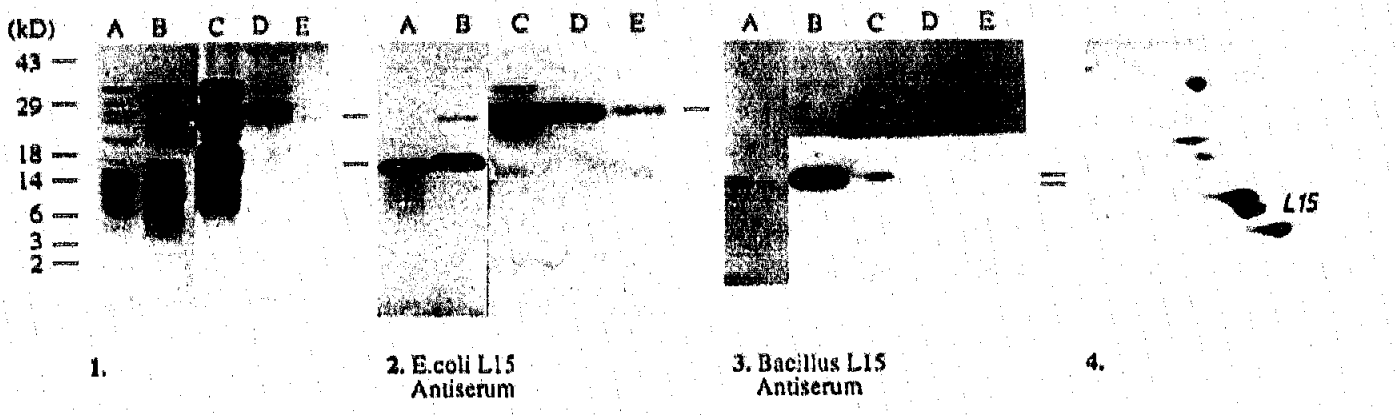


Fig. 4. SDS-PAGE, immunostained Westerns, and 2D-gel electrophoresis. In 1-3: (A) *E. coli* TP50; (B) *B. stearothermophilus* TP50; (C) spinach chloroplast TP50; (D) pool 45 proteins; (E) purified chloroplast L15. (1) and (4) are Coomassie-stained gels. (2) and (3) are immunostained Westerns of gel 1 using, respectively, anti-serum to *E. coli* L15 or *Bacillus* L15; (4) is a 2D-gel of 6 μg of pool 45 proteins. $M_r$ standards are indicated to the left of the figure.

institute) databanks, no significant homology with any protein was found.

Internal sequence data were obtained from automated N-terminal sequencing of peptide fragments derived from a Lys-C endoprotease digestion. The peptides were purified by RP-HPLC (Fig. 1B). A total of 6 peptides were collected from the column, of which 5 (numbered in Fig. 1B) yielded unique sequences which totalled 93 amino acid residues (Fig. 2). Comparison of the C-terminal 10 residues of peptide 1 and the sequence of peptide 2 with the known protein sequences in the databanks indicated the purified protein as the L15 homologue of *E. coli*.

### 3.2. *Peptide alignments with bacterial L15s*

Fig. 3 shows the alignment positions for peptides 1, 2, 3 and 4 with *E. coli* [14,15] and *B. stearothermophilus* L15 [16]. The 80% identity of peptide 2 with the Arg-41 to Pro-62 region of *E. coli* L15 confirms the spinach chloroplast protein to be the L15 homologue. The 50% identity of the carboxyl 10 residues of peptide 1 with the N-terminal sequences of *E. coli* and *B. stearothermophilus* L15 (as well as a 40% invariance between the 3 sequences) leads to the conclusion that spinach L15 has an unusually serine-rich 18 residue N-terminal extension, S-A-S-S-S-N-V-S-P-S-I-G-S-G-S-E-T-R.

Although peptide 2 and the carboxyl 10 residues of peptide 1 aligned well with the *E. coli* L15 protein, peptides 3 and 4 showed various significant alignments. A number of these could be eliminated due to overlaps into the domains of peptides 1 and 2, low computer alignment scores, and/or the presence of gaps. The final alignments in Fig. 3 were selected on the basis of the highest scores and identities maintained among all 3 proteins. Peptide 3 aligned best with residues Asn-99 to Ala-113. It has only 3 identical residues with *E. coli* L15 but one of them is found in the same position in all 3 proteins. Peptide 4 aligned best with residues Val-127 to Lys-141. There are 5 identical residues with *E. coli* and *Bacillus* L15 (47% identity).

Peptide 5 showed a number of alignments but none with more than two identical residues with *E. coli* L15 and none identical in all 3 proteins. It was therefore inferred to be part of a spinach L15 insertion to the L15 homologous domain or a C-terminal extension.

These results indicate spinach chloroplast L15 has a domain with an overall primary structure similar to that of *E. coli* and *B. stearothermophilus* L15. The first half of all 3 molecules have very high identity (70%), but the latter part has less (29% identity). Thus, if more similarity is maintained in the carboxyl part it would be found in the secondary/tertiary structure rather than in sequence. The Chou and Fasman program predicts a similar overall secondary topology for both bacterial L15s [15].

### 3.3. *Immuno cross-reaction/Western blotting*

Similarities in primary structure as well as secondary/tertiary structure (in the event of conservative changes in primary structure) are detected by antibody cross-reactions [17]. Immunological cross-reactivity of chloroplast L15 with *E. coli* and *B. stearothermophilus* L15 antisera was therefore tested to determine further similarities in these molecules.

As demonstrated in Fig. 4 *E. coli* L15 antiserum shows expected specificity for the *E. coli* protein of molecular mass of 15 kDa (14 983 Da from the sequence [15]). The serum shows cross-reaction with a single protein in *B. stearothermophilus* TP50, and with a protein, $M_r$ 22 000, in the chloroplast TP50. This confirms again the identity and the significant increase in mass of the spinach L15 protein over its *E. coli* counterpart. A cross-reacting protein of identical molecular mass is detected in the pool 45 proteins and with the purified spinach L15 protein. 2-D gel analysis of the pool 45 pro-

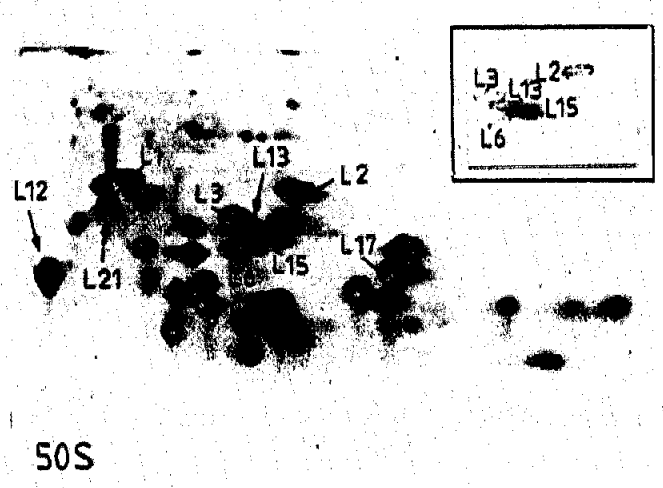


Fig. 5. Two-D gel of spinach chloroplast 50S subunit proteins [19] showing the location of L15. The inset is from a similar 50S 2-D gel that was Western blotted and immunostained with *E. coli* L15 antiserum. The locations of other cross-reacting L-proteins [7,8] and L21 [2] are also shown.

teins shows there is only one protein of this molecular mass in the pool (Fig. 4.4).

These results and the data on sequence identity confirm the assignment of this spinach protein as the *E. coli* L15 homologue. The increase in size of the L15 protein (~7000 Da) can only in part be accounted for by the N-terminal 18-residue extension (1700 Da calculated from the sequence). Thus the presence of a significant C-terminal extension or an insertion(s) within the *E. coli* homologous domain is also confirmed.

The significant cross-reactivity of *Bacillus* and spinach chloroplast L15s with *E. coli* L15 antiserum would indicate an antiserum for any of these 3 should cross-react with the others. Therefore, an experiment was performed with a *B. stearothermophilus* L15 antiserum. Fig. 4.3 shows the specificity of this antiserum for the *Bacillus* L15. Surprisingly, it has considerably less cross-reactivity for the *E. coli* L15 (lane A) and little to no cross-reactivity for the chloroplast L15 (lanes C, D and E).

### 3.4. *2D-gel location of L15*

Immunostaining of a 2D gel Western of chloroplast TP50 with the *E. coli* L15 antiserum confirmed the location of L15 in the 2D gel pattern of spinach chloroplast 50S subunit proteins. The results together with the locations of other immunologically cross-reacting proteins [7,8] and L21 [2] are presented in Fig. 5. Including L15 (this work) and the chloroplast encoded protein L2 [10,18] the total number of such cross-reacting spinach chloroplast 50S r-proteins is presently 8 (L1, L2, L3, L6, L12, L13, L15, L17).

## 4. DISCUSSION

The purification, identification and characterization of the spinach chloroplast L15 protein is described in this paper. The amino acid sequence of several internal peptide fragments and immunological homology of the protein with *E. coli* L15 are also shown. The data reveal the presence of an 18 residue N-terminal extension, and a larger C-terminal extension to (or insertions within) the *E. coli* homologous domain of spinach L15. Furthermore, the lack of any peptide identity with the coding sequences from the complete chloroplast DNA of tobacco, rice, and liverwort [9] imply that this protein is encoded within the plant nuclear DNA. A pea L15 cDNA clone has been recently isolated and characterized (personal communication, J.S. Gantt, University of Minnesota). Since chloroplast r-proteins encoded in the nuclear DNA are synthesized as cytoplasmic precursors, the N-terminal sequence of L15 presented here gives the precise processing site of the spinach L15 precursor.

Three spinach chloroplast proteins, L1 [8], L13 [1] and L21 [2], have been shown to maintain increased mass over their *E. coli* counterparts. Complete characterization of L13 and L21 showed this increase due primarily to N-terminal and C-terminal extensions [1,2]. Spinach chloroplast L3 also has ~1 kDa increased mass over its *E. coli* counterpart, the increase being due to an N-terminal extension (C. Johnson, unpublished data). Since none of the characterized spinach chloroplast r-proteins has large insertions within the *E. coli* homologous domain (which could disrupt any similar secondary structure therein), it is likely that the 'extra' ~5000 Da increased mass in the chloroplast L15 is also at the C-terminus.

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