

Seasonal expression patterns and characterization of a *Euphorbia esula* root storage protein

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Abstract High-abundance, water-soluble root proteins are hypothesized to be potential storage forms of reduced N assimilate in perennial weeds. Rabbit antiserum raised against an electrophoretically purified 29 kDa root protein of leafy spurge (*Euphorbia esula* L.) was used to follow seasonal protein accumulation patterns as well as responses to photoperiod and decapitation. Accumulation of the protein in roots of field-grown plants began during late spring months, with maximal accumulation observed during fall and winter months. Protein accumulation patterns over an 8-week period were similar in root extracts from controlled-environment long- and short-day-grown plants at 30°C. Decapitation of the shoot resulted in an immediate decline of the 29 kDa protein in the roots, followed by a gradual increase as shoots expanded from root buds over a 4-week period. These accumulation patterns suggest that dormant leafy spurge root buds may exploit the degradation of stored N root reserves in the form of soluble storage proteins for amino acids to fuel expansion and growth when the buds are released from apical dominance and the root undergoes a transition from sink to source. Processes controlling the accumulation or remobilization of such perennial root storage proteins thus provide potential targets for chemical or biological weed control agents.

Key words Perennial weed, vegetative storage protein, *Euphorbia esula*.

Abbreviations BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IgG, Immunoglobulin-G class; LD, long day; LSRP 29, 29 kDa leafy spurge root protein; PAGE, polyacrylamide gel electrophoresis; %T, % total bis-acrylamide + acrylamide monomers; PMSF, phenylmethylsulfonyl fluoride; PVPP, polyvinylpyrrolidone; SDS, sodium dodecyl sulfate; SD, short day; TBS, Tris-buffered saline (25 mM Tris-HCl, pH 7.2, 150 mM NaCl).

INTRODUCTION

Leafy spurge (*Euphorbia esula* L.) is a noxious perennial weed which has rapidly spread across the Northern plains states of the U.S. by both asexual clonal reproduction and seed dispersal. The asexual reproductive strategy of perennial weeds such as leafy spurge is exceptionally problematic in that clonal vegetative reproduction from root and crown buds tend to form dense monogenotypic stands which are

expensive to control with chemical herbicides on large rangeland acreages (Messersmith *et al.*, 1985). Root and crown buds develop underground throughout the growing season and remain dormant until apical dominance is released by senescence or defoliation (Raju *et al.*, 1964). Once dormancy is released, the buds expand aboveground into photosynthetically active shoots unless restrained by low temperatures in winter (Schimming and Messersmith, 1988). A short period of low temperature is required before

seasonal release of bud dormancy in spring (Harvey and Nowierski, 1988).

Leafy spurge stores substantial quantities of carbon (as starch) and nitrogen reserves (as free amino acids and soluble proteins) (Cyr and Bewley, 1989) in the storage parenchyma of primary roots (Raju *et al.*, 1963). McIntyre and Raju (1967) postulated that nitrogen reserves may be a limiting factor in growth of root and crown buds during spring regrowth from leafy spurge roots. This hypothesis was strengthened by the finding of Cyr and Bewley (1990a) that putative storage proteins with distinct seasonal accumulation patterns were present in roots of leafy spurge. Cyr and Bewley (1990a) postulated that stored free amino acids and storage proteins may supply root buds with the nitrogen needed for rapid growth during spring months or upon removal of apical dominance. Thus, an understanding of the factors controlling expression and degradation of such proteins is an important component to a long term control strategy for the weed. Should root storage proteins prove to be N storage sinks required for bud growth in roots of perennial weeds such as leafy spurge, they would provide attractive biochemical targets for weed control.

We have targeted a prominent 29 kDa buffer-soluble protein of leafy spurge roots (LSRP 29) for intensive investigation as to the potential role of proteins in the nitrogen storage of the root. This report details the characteristics and accumulation patterns of this putative storage protein in leafy spurge root tissues.

RESULTS

Monthly variation in leafy spurge root protein patterns

SDS-PAGE profiles of buffer-soluble root proteins in leafy spurge collected from the field at monthly intervals from March 1993 through February 1994 exhibited seasonal accumulation patterns for several polypeptides. In particular, a 29 kDa polypeptide (LSRP 29) (*fig. 1*, arrow) was detected from June through January but not detectable by Coomassie staining in samples collected during March through May.

Soluble proteins were highest in root extracts collected from field-grown plants during the late fall and winter months (*fig. 2*). It should be emphasized that the SDS-PAGE gels represented in figure 1 were loaded with equal amounts of soluble protein, indicating the specific accumulation of LSRP 29 when total soluble protein levels in the root were low in late spring and early summer months (*fig. 2*).

Amino acid content of purified LSRP 29

SDS-PAGE gel-purified, electroeluted LSRP 29 was subjected to amino acid analysis. The LSRP 29 contained a high content of ASX and GLX, SER, GLY and ALA (*tab. 1*). No CYS was detected, consistent with the observation that elimination of reducing agents prior to SDS-PAGE had no effect on the mobility of the protein (data not shown).

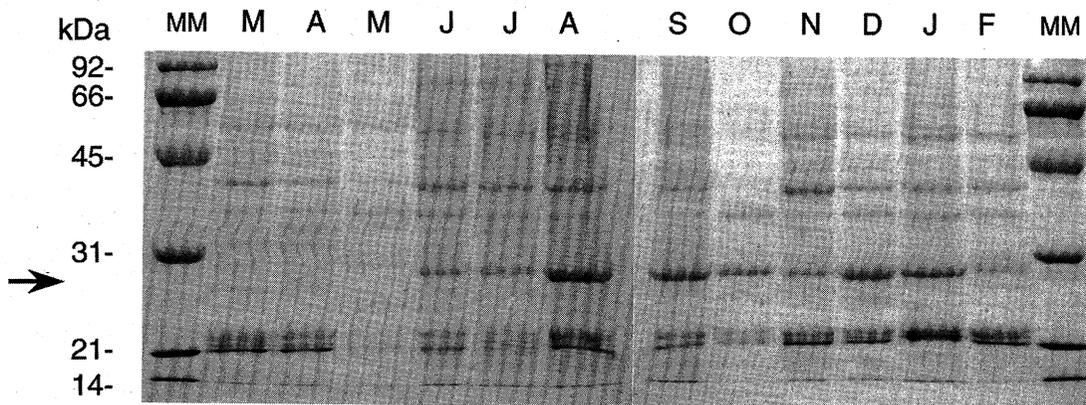


Figure 1. SDS-PAGE analysis of proteins in buffer extracts of field-grown leafy spurge collected at monthly intervals. Equal amounts (25 μ g) of extracted protein were loaded on 12% T mini-gels and visualized with Coomassie blue R-250 stain. Lanes are sequentially labeled with the abbreviation for the month in which the sample was collected. MM; molecular mass markers in kDa. The arrow points to the migration of LSRP 29.

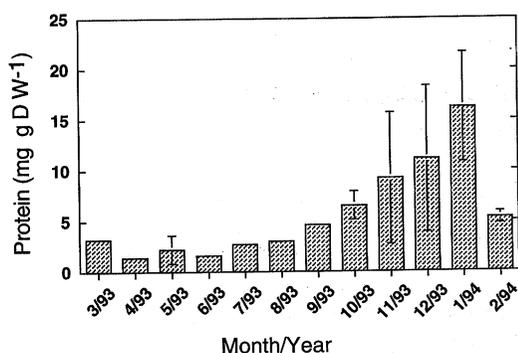


Figure 2. Total buffer-soluble protein in buffer extracts of field-grown leafy spurge collected at monthly intervals. Bar heights represent the mean of at least two separate plant samples. Error bars indicate standard deviation.

Table 1. Amino acid composition of the SDS-PAGE purified LSRP 29. The number of amino acid residues per LSRP 29 polypeptide chain was calculated from a normalized PHE content of 9.00 residues per chain corresponding to a predicted molecular mass of 29 kDa. Estimations are based on 5 determinations on the same batch of purified protein. (N.D. = not detected; N.S.; not significantly above background analysis in the original chromatogram).

Amino acid	Residues per chain
ALA	20.28
ARG	8.63
ASX	25.03
CYS	N.S.
GLX	32.05
GLY	29.28
HIS	5.05
ILE	12.33
LEU	22.68
LYS	16.27
MET	3.70
PHE	9.00
PRO	11.84
SER	26.95
THR	16.15
TRP	N.D.
TYR	9.99
VAL	17.14

N-Terminal amino acid sequence of purified LSRP 29

N-terminal sequence analysis of the SDS-PAGE-purified LSRP 29 indicated the consensus sequence; X E H V I F Y V I D P A A E S Y T K F L I T L R S for the first 25 amino acid residues at the N-terminus of SDS-PAGE-purified LSRP 29 deduced from two individual sequencer runs on the same batch of purified protein. No significant sequence identity was detected with other

plant vegetative or seed storage protein sequences upon searching the GenBANK/EMBL or SwissPROT protein sequence databanks using the LSRP 29 amino acid sequence translated to all possible nucleic acid sequences ("BACKTRANSLATE" and "FAST A", Genetics Computer Group, v.7, 1991, 575 Science Dr., Madison, WI 53711). No glycosylation motifs were apparent in the first 25 amino acids of LSRP 29, although preliminary results with a glycosylation Western blot detection analysis had indicated biotinylated concanavalin A binding to LSRP 29 (results not shown).

Characterization of antisera to LSRP 29

Anti-LSRP 29 preparations exhibited specificity for SDS-denatured LSRP 29 on Western blots, while undiluted preimmune sera did not react with any leafy spurge root proteins (data not shown). At higher (> 2 µg) protein loads on Western blots, polypeptides of ca 21, 26 and 28 kDa were weakly reactive with anti-LSRP 29 antisera (not shown). Protein A/G affinity purified anti-LSRP 29 IgG fractions exhibited high affinity to and retained high specificity for SDS-denatured LSRP 29.

Characterization of LSRP 29 expression patterns in response to photoperiod and shoot removal

Western blots of buffer-extracted, SDS-PAGE-separated leafy spurge root proteins collected from field-grown plants at monthly intervals were probed with a Protein A/G-Sepharose affinity purified anti-LSRP 29 IgG fraction. The blot (fig. 3) showed similar seasonal patterns of accumulation of LSRP 29 as those seen on Coomassie-stained SDS-PAGE gels (fig. 1). As in figure 1, SDS-PAGE gels for Western blots were loaded with equal amounts of protein (1.0 µg), indicating the accumulation of LSRP 29 even during periods of low soluble root protein content in late spring and early summer. Thus, LSRP 29 accumulates against the background of low total protein during late spring and summer months.

To examine the effect of photoperiod on LSRP accumulation, plants which had been decapitated and allowed to regenerate outside in July for three weeks were transferred to controlled environment growth chambers, under 8:16 (SD) and 16:8 (LD) h light:dark photoperiod regimes. Western blots of buffer-soluble proteins extracted at weekly intervals from roots of SD and LD plants probed with affinity purified anti-LSRP 29 indicated no change in the

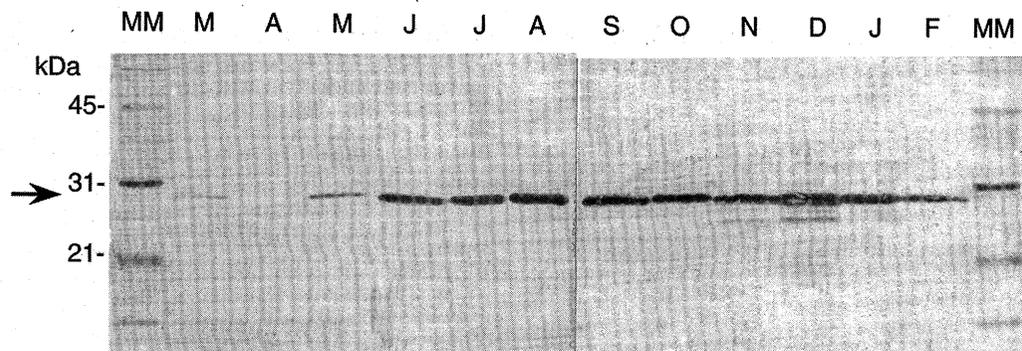


Figure 3. Western blot analysis of proteins in buffer extracts of field-grown leafy spurge collected at monthly intervals, probed with protein A/G-purified antisera to LSRP 29. One μg of soluble root extract was loaded in each lane. Lanes are sequentially labeled with the abbreviation for the month in which the sample was collected. MM; avidin-alkaline phosphatase-detected biotinylated molecular mass markers in kDa. The arrow points to the migration of LSRP 29. Blots show representative results from triplicate samples.

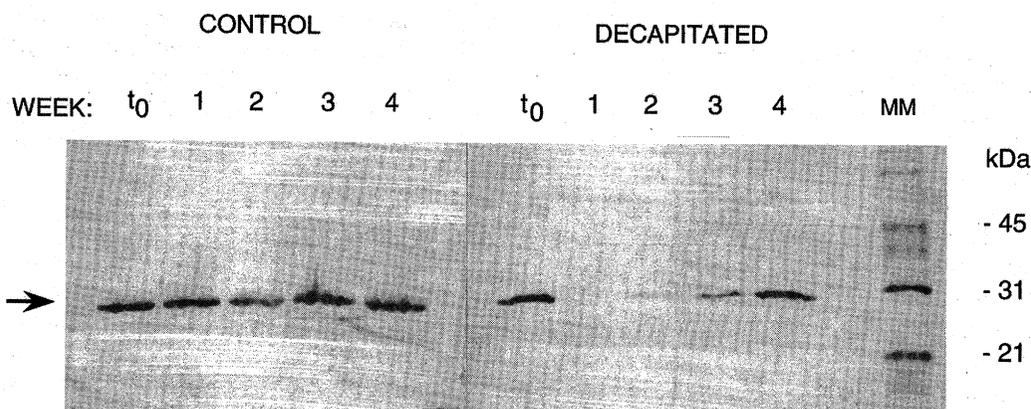


Figure 4. Western blot analysis of proteins in buffer extracts of roots of decapitated or control leafy spurge plants. One μg of soluble root extract was loaded in each lane. Lanes are sequentially labeled with the abbreviation for the week from t_0 at which samples were collected. MM; avidin-alkaline phosphatase-detected biotinylated molecular mass markers in kDa. The arrow points to the migration of LSRP 29. Blots show representative results from triplicate samples.

relative abundance of LSRP 29 over an 8-week period following transfer to SD or LD photoperiod regimes (data not shown).

To examine the effect of the release of apical shoot dominance and subsequent release of root bud dormancy on root LSRP 29 content, plants with initial, measurable levels of LSRP 29 were decapitated at the soil surface and allowed to regenerate new shoots. The plants had shoots with expanded leaves by two to three weeks following decapitation. Western blot analysis of LSRP 29 in roots over the 4 weeks following decapitation indicated a rapid decline in LSRP 29 within 1 week of decapitation compared to that present at the initiation of the experiment, followed by a gradual accumulation within 4 weeks (*fig. 4*). Control

plants expressed LSRP 29 at relatively constant levels throughout the experimental period.

DISCUSSION

We have isolated and characterized a 29 kDa protein that accumulates in root tissues of leafy spurge during periods of assimilative growth. LSRP 29 represents a large fraction of the soluble root protein during such growth periods, and is apparently degraded when N is required for regrowth of buds from root tissues. The LSRP 29 protein thus fulfills the definition of a vegetative storage protein (Staswick, 1994). Amino acid analysis indicated that LSRP contains a substantial proportion of GLX+ASX common to

many storage proteins. The overall low sulfur amino acid content is similar to that seen for other plant seed and vegetative storage proteins (Higgins, 1984) and explains our observation that the protein does not silver stain (data not shown). The protein is not particularly enriched in ARG or PRO, which are common storage protein amino acids, as measured for example in apple bark storage proteins (Tromp and Ovaa, 1973). The LSRP 29 N-terminal amino acid sequence is not similar to the sequence of any other known storage proteins. Although a possible role for LSRP 29 as an antifreeze or cold acclimation protein may be postulated based on its high abundance in late winter, the early accumulation of the protein in the summer months makes this hypothesis somewhat unlikely, unless the degree of glycosylation varies with season.

The LSRP 29 protein shows a similar accumulation pattern to a 26 kDa leafy spurge root protein seen on Coomassie-stained SDS-PAGE gels by Cyr and Bewley (1990a). While Cyr and Bewley (1990a) analyzed total protein patterns on Coomassie-stained SDS-PAGE gels, we utilized antibodies to focus on specific changes in LSRP 29 with increased sensitivity. The 26 kDa protein of Cyr and Bewley (1990a) and our 29 kDa protein (*fig. 1*) each migrate just below a bovine erythrocyte carbonic anhydrase molecular mass marker on SDS-PAGE gels of similar composition. We used a molecular mass marker protein mixture from BioRad (Richmond, CA) to estimate unknown protein molecular masses, including a putative 31 kDa bovine erythrocyte carbonic anhydrase molecular mass marker (*fig. 1*). Cyr and Bewley (1990a) used a molecular mass marker protein mixture from Sigma Chem Corp (St. Louis, MO) to estimate the sizes of leafy spurge root proteins, including a putative 29 kDa bovine erythrocyte carbonic anhydrase molecular mass marker. When we compared carbonic anhydrase markers obtained from BioRad and Sigma Chem Corp., the putative 29 and 31 kDa marker proteins migrated to equidistant points from the origin on SDS-PAGE gels (data not shown).

Thus, the 26 kDa leafy spurge root protein noted by Cyr and Bewley (1990a) and LSRP 29 probably represent the same protein. Both proteins accumulate from late spring to late winter, declining to near undetectable levels when root and crown buds expand in early spring. The observed declines in LSRP 29 protein seen in early spring may reflect both a decrease in protein synthesis rates and an increase in the rate of degradation of the specific protein by proteases in

the roots. Our results indicate that total soluble root protein peaked in late winter (*fig. 2*), declining in the spring, a trend also noted by Cyr and Bewley (1989) in leafy spurge root tissues.

It should be stressed that by loading equal amounts of protein on Western blots, the true LSRP 29 accumulation levels through time and various experimental conditions were determined. Thus, if viewed in the quantitative context of total protein present, LSRP 29 actually peaks with other soluble proteins in the late fall and winter months. The decline in soluble protein and the low LSRP 29 content seen in early spring correspond temporally to increased levels of free amino acids in the roots during spring months reported by Cyr and Bewley (1989). During this time, free amino acids are presumably transported to and incorporated into the expanding buds.

Seasonal patterns of accumulation and degradation have been observed for root storage proteins in the herbaceous perennials chicory, dandelion (Cyr and Bewley, 1990b) and alfalfa (Hendershot and Volenec, 1992), as well as for bark storage proteins in poplar (Clausen and Apel, 1991) and other hardwoods (Wetzel *et al.*, 1989; Arora *et al.*, 1992). While the expression of poplar bark storage proteins was demonstrated to be under strict short-day photoperiod control (Coleman *et al.*, 1991, 1992), we saw no difference in LSRP 29 accumulation patterns under long- or short-day photoperiods (data not shown). That the expression of LSRP 29 does not appear to be under photoperiod control is also evident in Western blots of monthly root extracts, which detected accumulation of LSRP 29 from June through December in field-grown plants (*fig. 3*).

Because decapitation of shoots leads to the release of dormancy and subsequent expansion of a finite number of root and crown buds, experiments were conducted to establish whether the removal of shoots affected the amount of LSRP 29 in the roots. Within one week of decapitation, LSRP 29 declined to undetectable levels in the root (*fig. 4*). The accumulation of the protein proceeded as shoots became autotrophic and, presumably, assimilation of reserves commenced. This response was in contrast to that observed by Cyr and Bewley (1990a) who followed soluble root protein SDS-PAGE patterns in decapitated, field-grown leafy spurge. They observed no differences in soluble protein patterns in roots of field-grown plants in the first two months following decapitation, but saw long-term inhibition of root protein accumulation in ensuing winter months. Our results, combined with

monthly temporal accumulation patterns and the lack of strict photoperiodic control of accumulation of LSRP 29, suggest that the accumulation of the protein is controlled by the source/sink status of the root buds.

The release of apical dominance leading to the expansion of the root buds likely initiates degradation of the storage proteins in the root to provide free amino acids required for growth, as proposed by Cyr and Bewley (1989). The development of expanding buds into autotrophic shoots in turn leads to the assimilation of reserves and/or the termination of degradation and mobilization of reserves. This model agrees with the observations of McIntyre and Raju (1967) who noted that leafy spurge plants grown under N deficiency (and presumed deficient in stored N root reserves) had greatly reduced numbers of root buds released from apical dominance in response to shoot removal. McIntyre (1972) also reported a complete suppression of bud elongation upon decapitation of plants which had been grown under N deficiency. In agreement with these findings, Ourry *et al.* (1994) found a significant correlation between root N and shoot regrowth yield in nonnodulated *Medicago sativa*. They concluded that root N storage reserves were the determining factor in shoot yield in regrowth following decapitation, and further showed that root C reserves were not significant factor in shoot yield (Ourry *et al.*, 1994). Our results strengthen the hypothesis that dormant leafy spurge root buds depend upon degradation of stored N root reserves in the form of soluble proteins such as LSRP 29 for amino acids to fuel expansion and growth when the buds are released from apical dominance and the root undergoes a transition from sink to source. Processes controlling the accumulation or remobilization of such perennial root storage proteins thus provide potential targets for chemical or biological weed control agents.

METHODS

Plant material. Root material for monthly samples or growth chamber experiments was collected from field-grown leafy spurge (*Euphorbia esula* L.) plants in Frederick, MD that had been sown in 1989 from seed collected from a Kindred, North Dakota leafy spurge infestation in 1988. Clones for growth chamber experiments were generated from multiple root cuttings of a single field plant. Root cuttings (5 cm long by 1-2 cm thick) were potted in a local clay-loam soil mix amended with sand (8%, w/w) peat moss, vermiculite and perlite (16% each w/w). Potted plants were grown outdoors in June and July prior to placement into

growth chambers, and were watered twice daily and fertilized biweekly with 20-20-20 (% w/w) NPK liquid fertilizer (Peters, Grace Sierra Products, Milpitas, CA). (The mention of vendor or product does not imply that they are endorsed or recommended by U.S. Department of Agriculture over vendors of similar products not mentioned).

Controlled environment experiments. Replicate plants were split into equal groups and placed in constant temperature and humidity growth chambers at 30°C under 16:8 h (light:dark) photoperiod regimes. For photoperiod experiments, separate plants were placed under identical conditions but under a 8:16 h (light:dark) photoperiod regime. Illumination in growth chambers was measured at 250-300 $\mu\text{E m}^{-2} \text{s}^{-1}$ at bench height 2 m from combined tungsten plus cool white fluorescent bulbs with a hand-held photometer (Li-Cor, Lincoln, NE). For decapitation experiments, plants were decapitated at the soil surface and new shoots allowed to regenerate from root or crown buds under a 16:8 h (light:dark) photoperiod regime. Plants were sampled and protein extracted from roots before decapitation and every week thereafter for 4 weeks. The plants had shoots with expanded leaves by two weeks following decapitation.

Sampling methods. Plants from monthly outdoor sampling or growth period experiments were harvested into ice water, washed with 18 Mohm ultrapure water and excised root segments were divided into 50 ml plastic sterile tubes. Root tissue was rapidly frozen in liquid nitrogen, lyophilized to dryness, milled through a 40 mesh screen and stored at -20°C prior to protein extraction. Three plants were sampled, extracted and analyzed separately at each time point in each experiment. Western blots show representative results from triplicate samples.

Protein extraction. Milled, lyophilized tissue (50 mg) was extracted in a 2 ml microcentrifuge tube for 5 min at 4°C in 1.5 ml of a buffer containing 50 mM $\text{KPO}_4\text{-KOH}$, pH 7.0, 50 mg PVPP, 5 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 μM pepstatin, 2 μg aprotinin ml^{-1} and 100 μM each leupeptin and chymostatin with gentle rocking. The slurry was spun in a microcentrifuge at 4°C with a horizontal microcentrifuge tube rotor (Sorvall SH-MT, DuPont Sorvall, Wilmington, DE) for 20 min at 10,000 $\times g$. The resulting supernatant was passed through a 0.45 μm nylon microcentrifuge filter (Millipore, Bedford, MA) and stored at -20°C.

Preparative SDS-PAGE and LSRP 29 purification. Protein extracts were dialyzed against 62 mM Tris-HCl (pH 6.8), and concentrated in 10 kDa cutoff membrane microcentrifuge filters (Amicon, Beverly, MA). Samples were brought to 5 mM DTT, 3% (w/v) SDS, 10% (v/v) glycerol and 0.05% (w/v) bromophenol blue and heated to 95°C for 5 min. Concentrated protein extracts were run on 3 mm thick preparative denaturing 12% T polyacrylamide slab gels (Laemmli, 1970) in a vertical mini-format

(6 × 8 cm) apparatus (Bio-Rad, Richmond, CA) at 200 V for 45 min. Proteins were localized by immersion of the gel slab in 50 mM KCl at 4°C, and the 29 kDa band was excised with a scalpel blade. Excised bands were equilibrated in 12.5 mM Tris-glycine (pH 8.0), 0.1% SDS electroelution buffer for 15 min and electroeluted (Isco, Inc., Lincoln, NE) with buffer circulation at room temperature (20-25°C) for 15 h at 15 mA per sample. Electroeluted proteins were reanalyzed on 12% polyacrylamide slab gels stained with Coomassie blue for purity evaluation and quantitated against a series of μg amounts of BSA on the gels.

Production and purification of antisera to LSRP 29. SDS-PAGE-purified LSRP 29 polypeptide was used to immunize New Zealand rabbits for production of antisera reactive against SDS-denatured LSRP 29. Antisera production was contracted to a commercial producer (Cocalico, Reamstown, PA). Anti-LSRP 29 IgG was purified by affinity chromatography on Sepharose-immobilized recombinant fusion protein A/G according to the manufacturer's protocols (Pierce Chem. Corp., Rockford, IL).

Analytical SDS-PAGE. Protein extracts were precipitated with trichloroacetic acid and deoxycholate by the method of Bensadoun and Weinstein (1976) for analytical SDS PAGE, and 25 μg equivalent of precipitated proteins were separated on 1 mm thick 12% T polyacrylamide mini-gels (Bio Rad, Richmond, CA) in Laemmli (1970) buffers with subsequent Coomassie blue R-250 staining.

Western blotting and immunochemical detection. Protein extracts were separated on 12% T single percentage or 10-20% T gradient SDS-PAGE mini-gels using Laemmli (1970) buffers. Proteins were transferred to 0.2 μM pore size nitrocellulose membranes in transfer buffer (Towbin *et al.*, 1979) at 30 V for 15 h, with prestained molecular mass standards (BioRad, Richmond, CA) included on gels to assess transfer efficiency. After transfer, blots were blocked in 3% (w/v) gelatin in TBS for 2 h, and subsequently probed with Sepharose-protein A/G-purified anti-LSRP 29 IgG at 1:2000 dilution in 3% (w/v) gelatin in TBS for 2 h. Blots were washed for 15 min twice in 100 ml TBS-0.05% Tween-20, followed by 15 min in 100 ml TBS. Blots were then probed with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chem. Co., St. Louis, MO) at 1:30,000 dilution in 3% (w/v) gelatin in TBS for 1 h. Avidin conjugated to alkaline phosphatase was included at 1:3,000 dilution to detect biotinylated molecular mass standards (Bio-Rad, Richmond, CA). Alkaline phosphatase was detected with kit reagents according to the manufacturer's protocols (Bio-Rad, Richmond, CA).

Protein assays. Protein was estimated by the method of Markwell *et al.* (1981).

Amino acid analysis. SDS-PAGE purified, electroeluted LSRP 29 was dialyzed overnight against 1000 vol of

TBS at 4°C. The protein was then exhaustively dialyzed against 3 changes of 1000 vol of 18 Mohm ultrapurified water for 24 h and subsequently stored at -20°C at a concentration of 0.2 $\mu\text{g ml}^{-1}$. Aliquots were placed in analysis tubes which had been pyrolyzed at 500°C, and dried in a Waters PicoTag work station (Waters-Millipore Corp., Milford, MA.). Hydrolysis was carried out in gas phase with 6 N HCl containing 1.0 % (v/v) phenol. The amino acids liberated were quantitated as their phenylthiocarbonyl derivatives using the Waters PicoTag HPLC system (Waters, Milford, MA.).

Protein microsequencing. The N-terminal amino acid sequence of the SDS-PAGE purified, electroeluted and exhaustively dialysed LSRP 29 was determined by automated Edman degradation on a pulse liquid sequencer with on-line phenylthiohydantoin amino acid analysis (Applied Biosystems 473A, Foster City, CA).

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