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Original Article

Purification and localization of p10, a novel protein that increases in nymphal regenerating legs of *Periplaneta americana* (American cockroach)

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ABSTRACT Content of a protein with a molecular mass of 10 kDa (p10) was found to increase significantly in regenerating legs of the American cockroach *(Periplaneta americana)*. This protein was purified to homogeneity from a homogenate of regenerating legs. Partial amino acid sequencing indicated that p10 is a novel protein. Immunoblotting showed that its content in regenerating legs was 30 times that in normal legs, and decreased significantly when leg regeneration was complete. An immunofluorescence study revealed that p10 localizes exclusively on the external side of newly formed epidermis of regenerating legs.

KEY WORDS: *10 kDa protein (p10), leg regeneration, epidermis, cockroach*

Introduction

The molecular mechanisms involved in regeneration of lost tissues or organs are one of the most attractive subjects in developmental biology, and regenerations of newt lenses (Agata *et al.*, 1985; Itoh and Eguchi, 1986), amphibian limb blastemas (Onda *et al.*, 1990) and mammalian liver (Nakamura *et al.*, 1989; Kinoshita *et al.*, 1991) have been studied extensively. In the nymphal stage, the cockroach can regenerate eyes (Hyde, 1972; Kunkel, 1985), antennae (French and Domican, 1982; Kunkel, 1985) and legs (Bohn, 1974; Kunkel, 1985): after their amputation complete legs are regenerated within a single moult (Kunkel, 1985). Grafting experiments have suggested the formation of two dimensional circular patterns secreted by epithelial sheets during reconstruction of appendages, but nothing is known about three dimensional pattern or the molecular mechanism involved (French, 1976; Bryant *et al.*, 1977; Truby, 1986).

Previously, we isolated two closely related C-type lectins named *Periplaneta* lectin (Kubo and Natori, 1987) and regenectin (Kubo *et al.*, 1990) from the hemolymph of the adult cockroach *(Periplaneta americana)*. Although both these lectins are normally present in the hemolymph, only regenectin appeared transiently in regenerating legs in the late stage of regeneration (Kubo *et al.*, 1990, 1991). In an immunofluorescence study regenectin was detected around developing muscle cells in regenerating legs, but was not detectable in nymphal legs harvested at various developmental stages (Kubo *et al.*, 1991). These results suggested differences in the process of regeneration and normal development of legs, and mobilization of some humoral components during regeneration (Kubo and Natori, 1987; Kubo *et al.*, 1990, 1991).

To gain more insight into the components specifically involved in leg regeneration in the cockroach, we examined proteins in homogenates of regenerating legs and found that a protein with a molecular mass of 10 kDa (p10) accumulates in regenerating legs. We purified this protein to homogeneity and found by immunofluorescence studies that in the regenerates it is on the external side of the epidermis.

Results

Purification of a 10 kDa protein from leg regenerates

Almost nothing is known about the molecular events involved in regeneration of cockroach legs. As a first step in studies on this process, we examined the total proteins in nymphal leg regenerates prepared at various times after amputation by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 1, two changes were apparent. A dense band corresponding to about 40 kDa protein (arrow 1), which is thought to be that of actin, and several other bands disappeared 11 days after amputation, but reappeared in the regenerated legs. In addition, a protein with a molecular mass of about 10 kDa (arrow 2) increased significantly after amputation, was maintained at high level throughout regeneration, and then decreased during the moult, only a very low level being detected in regenerated legs.

Abbreviations used in this paper: SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; FITC, fluoresceinisothiocianate.

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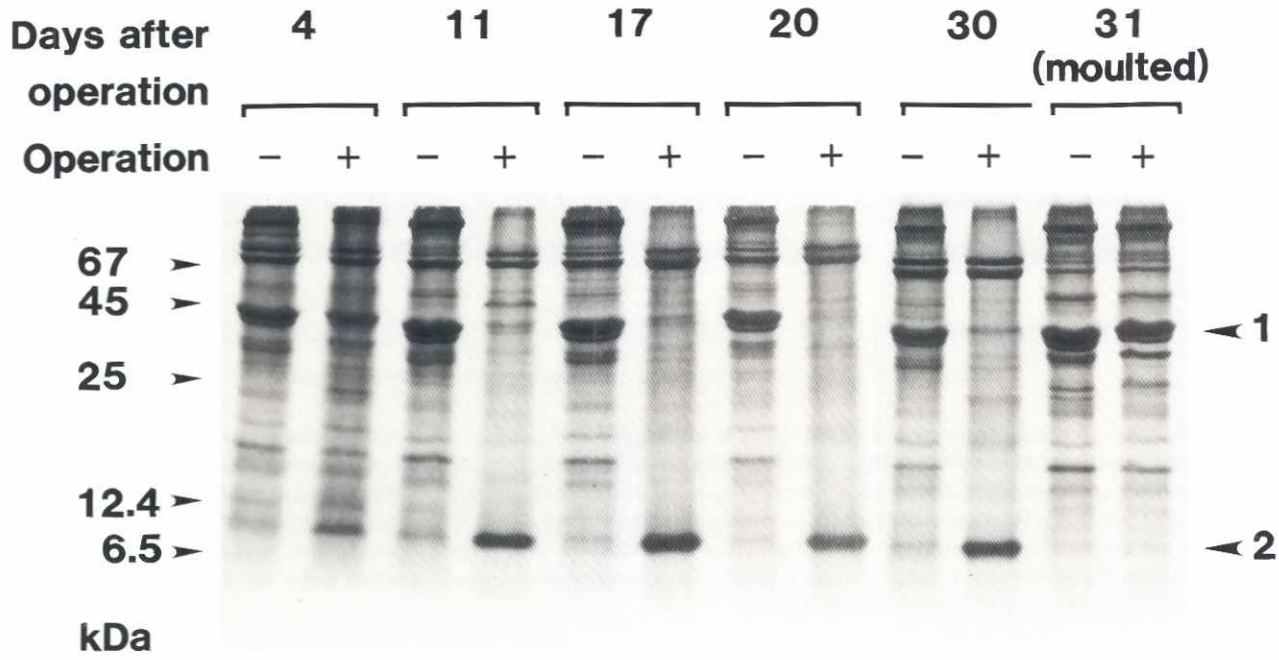

Fig. 1. Electrophoretic analysis of extracts of regenerating legs. *Nymphal regenerating legs (operation +) and control legs of the same animal (operation -) were harvested on the indicated days after amputation and homogenized, and soluble proteins were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Each lane contained 12 μg of protein. The molecular mass markers used for calibration of the gel were bovine serum albumin (67 kDa), ovalbumin (45 kDa), α-chymotrypsinogen (25 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). «Moulted» on day 31 indicates a completely regenerated leg.*

We were interested in this 10 kDa protein (p10), because it seems to be required at high concentration during leg regeneration, but at low level to maintain regenerated or normal legs. To gain more insight into the function of p10, we tried to purify it from homogenates of leg regenerates. For this, we subjected each fraction to SDS-polyacrylamide gel electrophoresis and monitored increase in the specific content of p10. We arbitrarily defined 1 unit of p10 as the amount giving the same density as 1 μg of bovine serum albumin on gel stained with Coomassie Brilliant Blue. The purification procedure included high speed centrifugation, and chromatographies on Sephacryl S-200 and Mono Q. The purification of p10 from about 150 regenerates harvested 11 to 30 days after amputation is summarized in Table 1, and the electrophoretic profile of the final Mono Q fraction is shown in Fig. 2. A major single band corresponding to 10 kDa protein was detected.

To confirm that this band was that of a single protein, we subjected this Mono Q fraction to HPLC on a reverse-phase C18 column. As shown in Fig. 3, a single symmetric peak was eluted from the column. Only this peak contained 10 kDa protein as shown by checking each fraction by SDS-polyacrylamide gel electrophoresis, and more than 99% of the applied protein was recovered in this peak. Therefore, we concluded that p10 was purified from leg regenerates to homogeneity by our procedure.

TABLE 1

SUMMARY OF PURIFICATION OF p10 FROM REGENERATING LEGS

Fraction	Protein (mg)	10 kDa protein (unit)	Specific content (unit/μg)	Purification (-fold)	Recovery (%)
Homogenate	37	4,400	0.12	1	100
100,000xg, sup.	20	3,400	0.17	1.4	77
Sephacryl S-200	0.98	1,700	1.7	14	39
Mono Q	0.24	780	3.3	28	18

Characterization of p10

Although the molecular mass of this protein was 10 kDa as determined by SDS- polyacrylamide gel electrophoresis, the native protein was a homodimer of 10 kDa subunits, because when the purified protein was subjected to a molecular sieve column of G3000SWxl calibrated with various molecular mass markers, it was eluted as a single peak of 18 kDa protein, as shown in Fig. 4.

We determined the sequence of the amino-terminal 28 amino acid residues and those of three peptides obtained by lysyl-endopeptidase digestion of p10 to be as shown in Fig. 5. No homologies were found between any of these sequences and those in data bases of the National Biomedical Research Foundation or GenBank, and no significant similarities were found with known amino acid sequences. We determined the amino acid composition of p10. As shown in Table 2, the contents of Lys and Leu were higher than those of other amino acid residues.

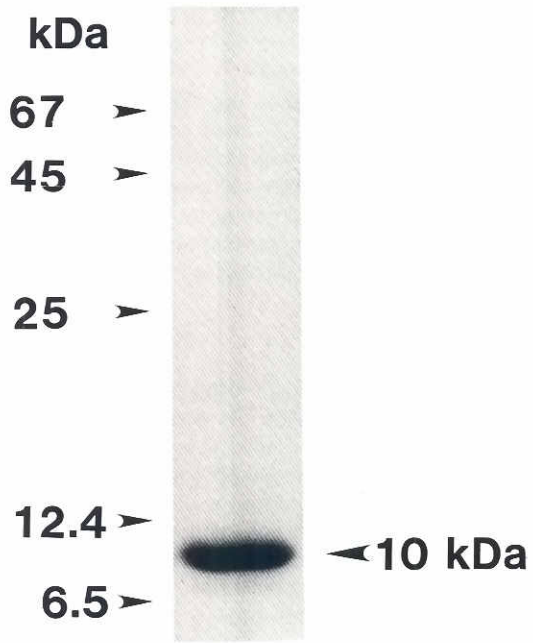

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified p10. *p10 recovered from Mono Q (1 μg) was subjected to SDS-polyacrylamide gel electrophoresis with the same molecular mass markers as for Fig. 1.*

TABLE 2

AMINO ACID COMPOSITION OF PURIFIED p10

Amino acid	Mol%
Aspartic acid	17.09
Threonine	5.15
Serine	4.91
Glutamic acid	10.94
Glycine	3.94
Alanine	6.57
Valine	2.92
1/2-Cystine	2.18
Methionine	0.88
Isoleucine	3.48
Leucine	10.64
Tyrosine	6.63
Phenylalanine	1.13
Lysine	13.92
Histidine	2.82
Arginine	2.91
Proline	3.06
Tryptophan	0.82

Pure p10 was hydrolized in 6 N HCl in the presence of 4% thioglycolic acid.

Immunoblotting analysis of p10

To gain insight into the function of p10 during regeneration, we raised antibody against p10, and quantitated the content of this protein in regenerates by immunoblotting. As shown in Fig. 6, a single band corresponding to purified p10 was detected when a homogenate of regenerates 21 days after amputation was subjected to immunoblotting, indicating that this antibody is specific for p10. Using this antibody, we analyzed the content of p10 in homogenates of regenerating legs harvested at various regeneration stages by immunoblotting. The amount of p10 in regenerates was calculated from a standard curve obtained with known amounts of p10.

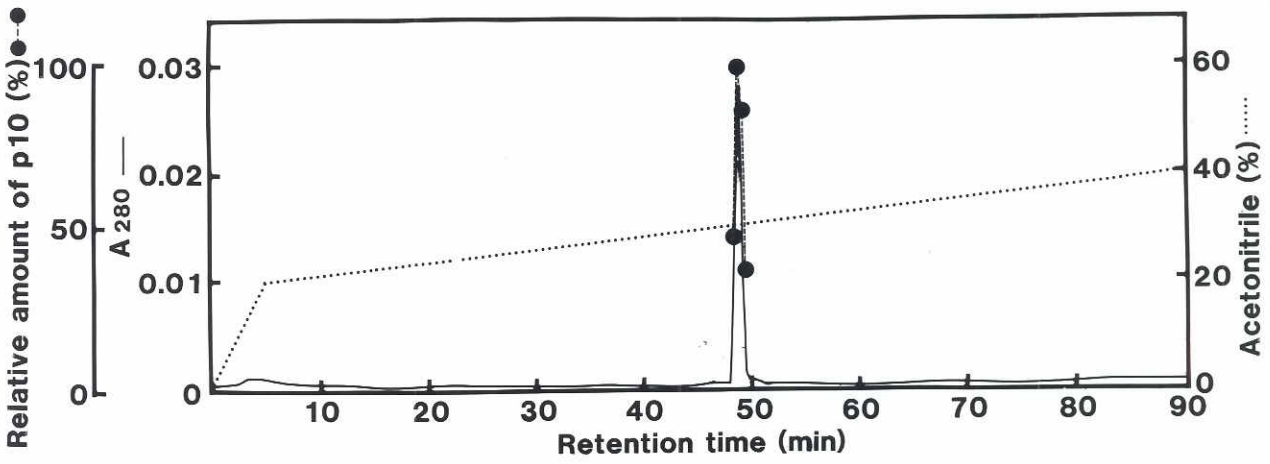

Fig. 3. Reverse-phase HPLC profile of p10. *Fractions from Mono Q were pooled (7.8 μg of protein) and subjected to HPLC on a Synchropak RP-P C18 column. A linear gradient of acetonitrile was formed with solution A (0.05% trifluoroacetic acid in H_2O) and solution B (0.05% trifluoroacetic acid in acetonitrile). Each fraction was lyophilized and subjected to SDS-polyacrylamide gel electrophoresis. ———, absorbance at 280 nm, ●—●, relative intensity of the band of p10 on the gel; ....., acetonitrile concentration.*

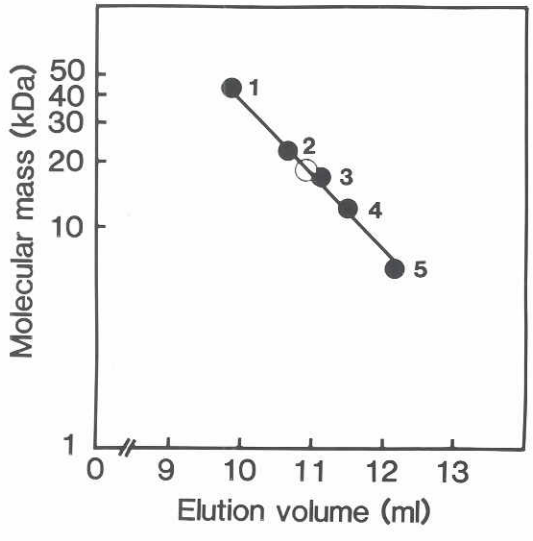

Fig. 4. Determination of molecular mass of intact p10. *Purified p10 (6.6 μg) was subjected to HPLC on a molecular sieve column of G3000SWxl with various marker proteins. Molecular mass is plotted against the elution volume. The following proteins were used for calibration of the column: 1, ovalbumin (45 kDa); 2, soybean trypsin inhibitor (22 kDa); 3, myoglobin (17 kDa); 4, cytochrome c (12.4 kDa); 5, aprotinin (6.5 kDa). The elution volume of p10 is shown by an open circle.*

As shown in Fig. 7, the content of p10 in regenerates was 20-30 times that in normal legs during the nymphal stage, but rapidly decreased to the control level after the moult, confirming the results of Fig. 1. A low basal level of p10 was always present in homogenates of control legs. Therefore, p10 is clearly not a specific protein for regenerating legs.

We examined whether p10 appears in adult legs with no regenerating ability after their amputation (Bodenstein, 1955).For this, the femur was first removed from the coxa, and then the remaining coxa was harvested at various times after the amputation and the content of p10 in the homogenate of coxa was measured. No significant increase in the content of p10 was detected. This finding suggests that p10 is needed for regeneration, not simply for wound healing of the amputated portion.

Localization of p10 in regenerates

Regeneration of nymphal legs after amputation proceeds as follows (Kunkel, 1985). Muscle in the coxa is partially degraded and a scab is formed over the wound. Then epidermal cells assemble and line up in the coxa to form the shape of the folded leg. Finally, myoblasts appear in the regenerating leg to construct muscle and cuticle surrounding the epidermis. We examined the localization of p10 in regenerates by an immunofluorescence study. Sections of the coxa containing a regenerating leg (26 days after amputation) were treated with affinity-purified antibody against p10, and the antibody was located with fluorescence-labeled second antibody. Strong nonspecific fluorescence was detected in the cuticle of the coxa, but specific fluorescence was observed only in the epidermis of the regenerating leg (Fig. 8A, B). A similar distribution of p10 was observed in coxae harvested on day 18 and day 30 after amputation. These results suggest that p10 is needed for formation of epidermis. We could not determine the localization of p10 in regenerates at much earlier stages.

The fluorescence tended to be more intense on the external side of newly formed epidermis. Fig. 9A shows a magnified field of newly formed epidermis, both the top and bottom parts being inside the regenerating leg. Nuclei of epidermal cells did not contain fluorescence, and fluorescence was intense on the external side of the layer of epidermal cells. It is uncertain whether this fluorescence was in the cytoplasm of epidermal cells or in the extracellular matrix.

Discussion

Nothing is known about the biochemical events involved in regeneration of cockroach legs. To understand the molecular mechanisms involved in regeneration, it is important to investigate the proteins that show dynamic changes in contents during regeneration. Previously, we reported that regenectin, a hemolymph C-type lectin, appears transiently around developing muscle cells in the late stage of leg regeneration, and we suggested that regeneration might be a systemic event (Kubo *et al.*, 1990, 1991). In this paper, we newly identified and purified a protein (p10) that increases significantly in regenerating leg. The function of this p10 is unknown, but we suggested by immunofluorescence studies that it located in newly formed epidermis, especially on the external side of the epidermis.

Immunofluorescence studies showed that p10 is clearly accumulated in the late stage of regeneration. However, no p10 was detected in fluorescence studies on days 4 and 10 after amputation, although significant p10 was detected in homogenates by immunoblotting. Therefore, p10 probably exists as a soluble form at early stages and gradually accumulates in the epidermis when epidermal cells line up during regeneration. The behavior of p10 in regenerates suggests that this protein participates in the formation of epidermis by controlling the assembly of epidermal cells.

p10 is a relatively abundant protein and its content is more than 5% of the total proteins in homogenates of regenerating legs. Therefore, it seems to be a structural protein constructing epider-

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          1    5        10   15   20   25
(1) DDKYTTKYDNIKLKEILASDRLXANYHK

          1    5
(2) VINFLYNK

          1    5    10
(3) KKPMWESLQK

          1    5    10
(4) KYDPENTYVTK
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Fig. 5. Partial amino acid sequences of p10. (1) *Amino acid sequence of the amino-terminal region. X indicates an unidentified amino acid.* **(2-4)** *Amino acid sequences of three peptides formed by digestion of p10 with lysyl-endopeptidase.*

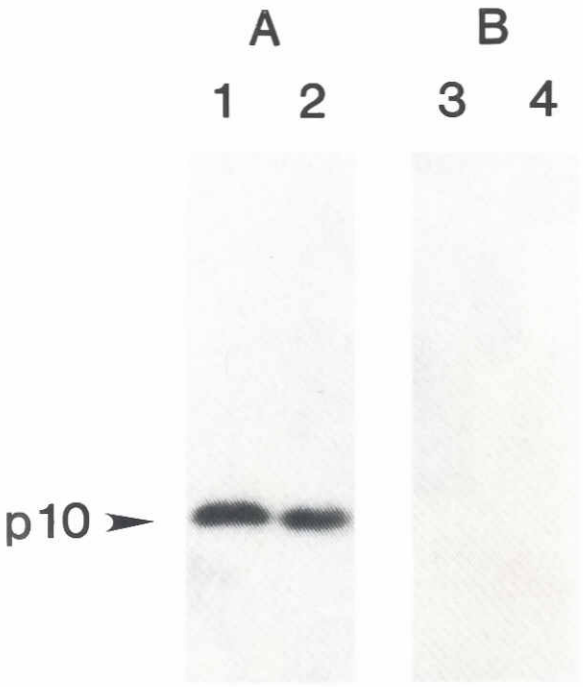

Fig. 6. Specificity of antibody against p10. *A homogenate of regenerating legs collected 21 days after amputation and purified p10 were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-p10 antibody* **(A)** *or preimmune serum* **(B)**. *Lanes 1 and 3, homogenate of regenerating legs (2 µg protein); lanes 2 and 4, purified p10 (50 ng).*

mis rather than an enzyme. Then, why does it disappear rapidly when regeneration is complete? Probably, p10 is present in an insoluble form in the final stage of regeneration for some reason such as cross-linking, and so cannot be detected by immunoblotting. Another possibility is that p10 is selectively degraded at this stage. We tried to localize it by immunofluorescence in regenerates at this stage, but nonspecific fluorescence of the cuticle is too strong to allow distinction of fluorescence of the epidermis.

Judging from its partial amino acid sequences, p10 seems to be a novel protein. The present study did not show whether p10 is an extracellular or intracellular protein, and cells producing it were not identified. Isolation of p10 cDNA is important for further characterization of p10.

Materials and Methods

Animals and preparation of regenerating legs

A colony of American cockroaches *(P. americana)* was maintained in a plastic container at 27°C with dog biscuits and water. Nymphs of 1.5-2.5 cm body length were collected 0-1 day after a molt, and anesthetized on ice. Then their left prothoracic and right metathoracic legs were amputated at the junction between the coxa and femur with fine scissors. The wound healed, leg regeneration proceeded in the amputated coxa, and a completely regenerated leg was formed by the time of the next moult (about 30 days after the operation). Regenerates were removed from the coxae at various times after the amputation for use in subsequent experiments. Corresponding portions of the right prothoracic and left metathoracic legs of the same animals were used as control intact legs.

Purification of p10

Regenerates collected from about 80 nymphs were homogenized in 8 ml of buffered insect saline (10 mM Tris-HCl buffer, pH 7.9, containing 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) in a glass homogenizer on ice. The homogenates were centrifuged at 700 x g for 10 min to remove debris, and the resulting supernatant was centrifuged at 100,000xg for 30 min. The supernatant was fractionated on a column of Sephacryl S-200 (1.8 x 66 cm) equilibrated with buffered insect saline. Fractions containing p10 were pooled (11.5 ml), and concentrated with an ultrafiltration YM-2 membrane (Millipore). The concentrated fraction was diluted with 4.5 ml of 10 mM Tris-HCl buffer, pH 7.9, containing 1 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$ (buffer A), and subjected to FPLC on a Mono Q column equilibrated buffer A. Material was eluted with a 10 ml linear gradient of 1-300 mM NaCl in buffer A. This protein was eluted as a single peak with 100 mM NaCl, and was almost homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

Electrophoresis and immunoblotting

Electrophoresis in SDS-polyacrylamide slab gel was carried out by the method of Laemmli (1970). Samples were denatured by heating them for 20 min at 75°C in 1% (w/v) SDS and 2% (v/v) 2-mecaptoethanol. After electrophoresis, the gels were stained by the method of Fairbanks *et al.* (1971).

Proteins separated by electrophoresis were transferred electrophoretically to a polyvinylidene difluoride filter (Immobilon, Millipore), and the filters were washed for 15 min at room temperature with rinse solution (10 mM Tris-HCl buffer, pH 7.9 containing 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.01% sodium azide and 0.25% skim milk) to remove SDS. Then the filters were immersed in skim milk solution (20 mM Tris-HCl buffer, pH 7.9 containing 5% skim milk) for at least 1 h, transferred to 5 ml of rinse solution containing antiserum against p10 (diluted 2,000-fold) and shaken gently for 12 h at 4°C. They were then washed well with rinse solution, transferred to 10 ml of rinse solution containing radioiodinated anti-rabbit Ig (37 kBq) and shaken gently for 4 h at room temperature. Finally they were washed well with rinse solution, dried and autoradiographed with Kodak XAR film.

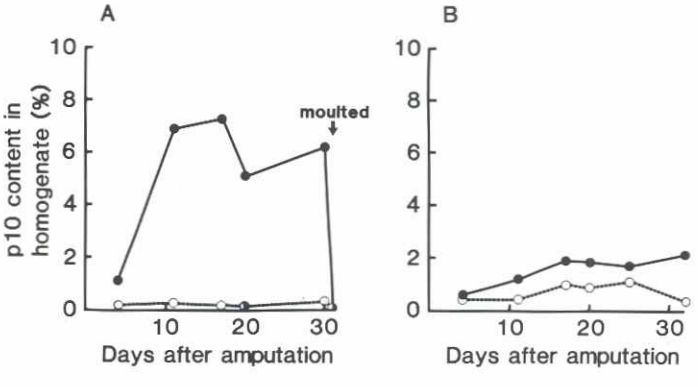

Fig. 7. Change in the amount of p10 in legs after amputation. (A) *Regenerates of nymphal legs and control legs of the same animal were collected various times after amputation, and their homogenates were subjected to immunoblotting with antibody against p10. The amount of p10 in the homogenate was determined from the intensity of the band of p10 on the autoradiogram using a standard curve obtained with known amounts of p10. The p10 content of the homogenate was calculated as a percentage of the total amount of protein.* **(B)** *The same experiment was performed with adults. After amputation of legs, wound healing took place, but no leg regeneration was detected.* ●—●, *amputated legs;* ○---○, *control legs. «Moulted» in (A) indicates completely regenerated legs of moulted animals on day 31.*

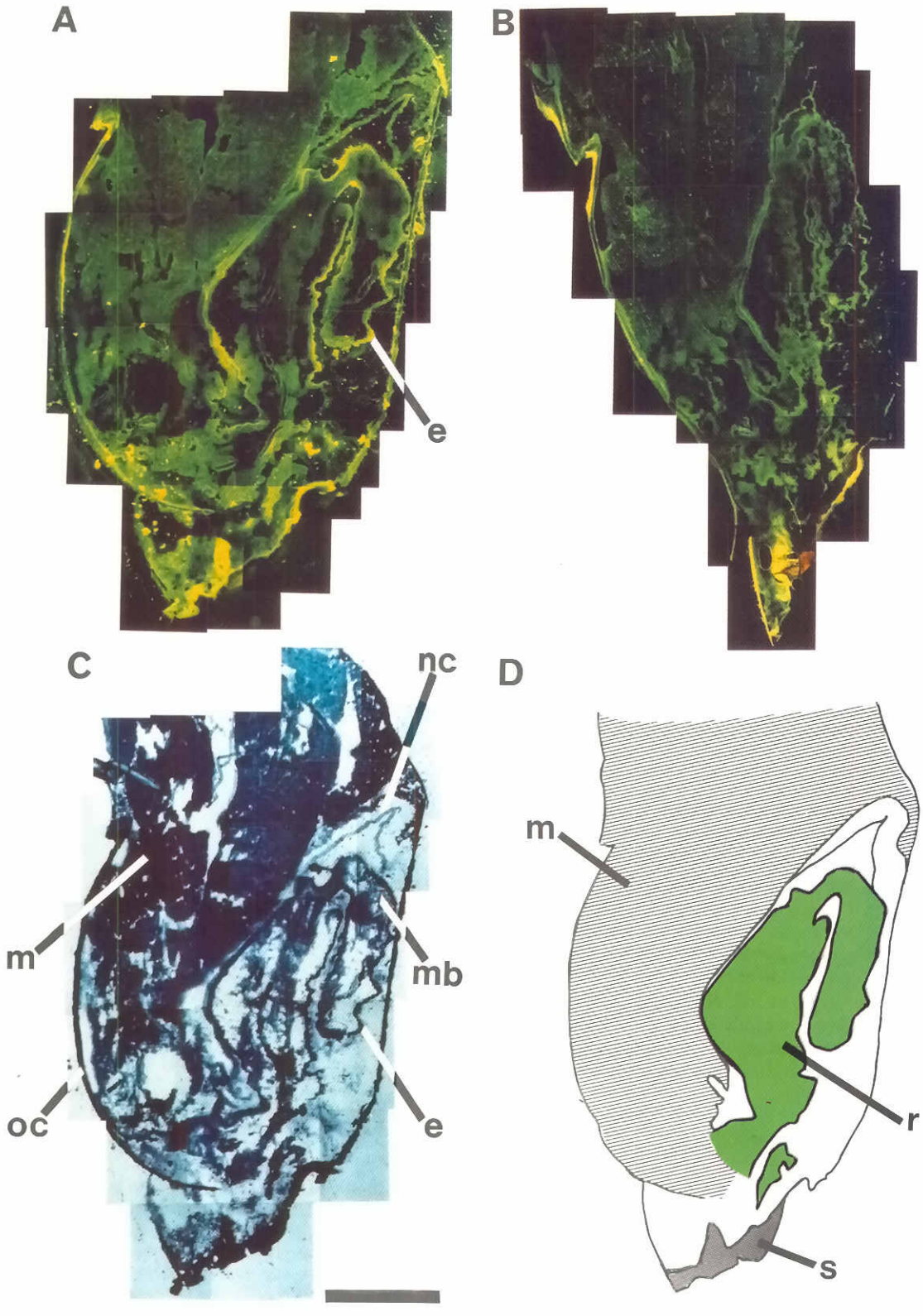

Fig. 8. Localization of p10 in a regenerate on day 26. *Thin sections (10 μm each) of the regenerate were prepared and subjected to immunofluorescence staining with affinity-purified antibody against p10.* **(A)** *Immunofluorescence of p10 antibody.* **(B)** *Background fluorescence of a consecutive section to that in (A) obtained with the same amount of normal antibody.* **(C)** *Toluidine blue staining of the section shown in (A).* **(D)** *Schematic illustration of the regenerate. Symbols: e, epidermal cells of the regenerate; nc, premature new cuticle of regenerate; mb, myoblasts of regenerate; oc, old cuticle of coxa; m, muscle of coxa; s, scab; and r, regenerating leg. Bar indicates 0.3 mm.*

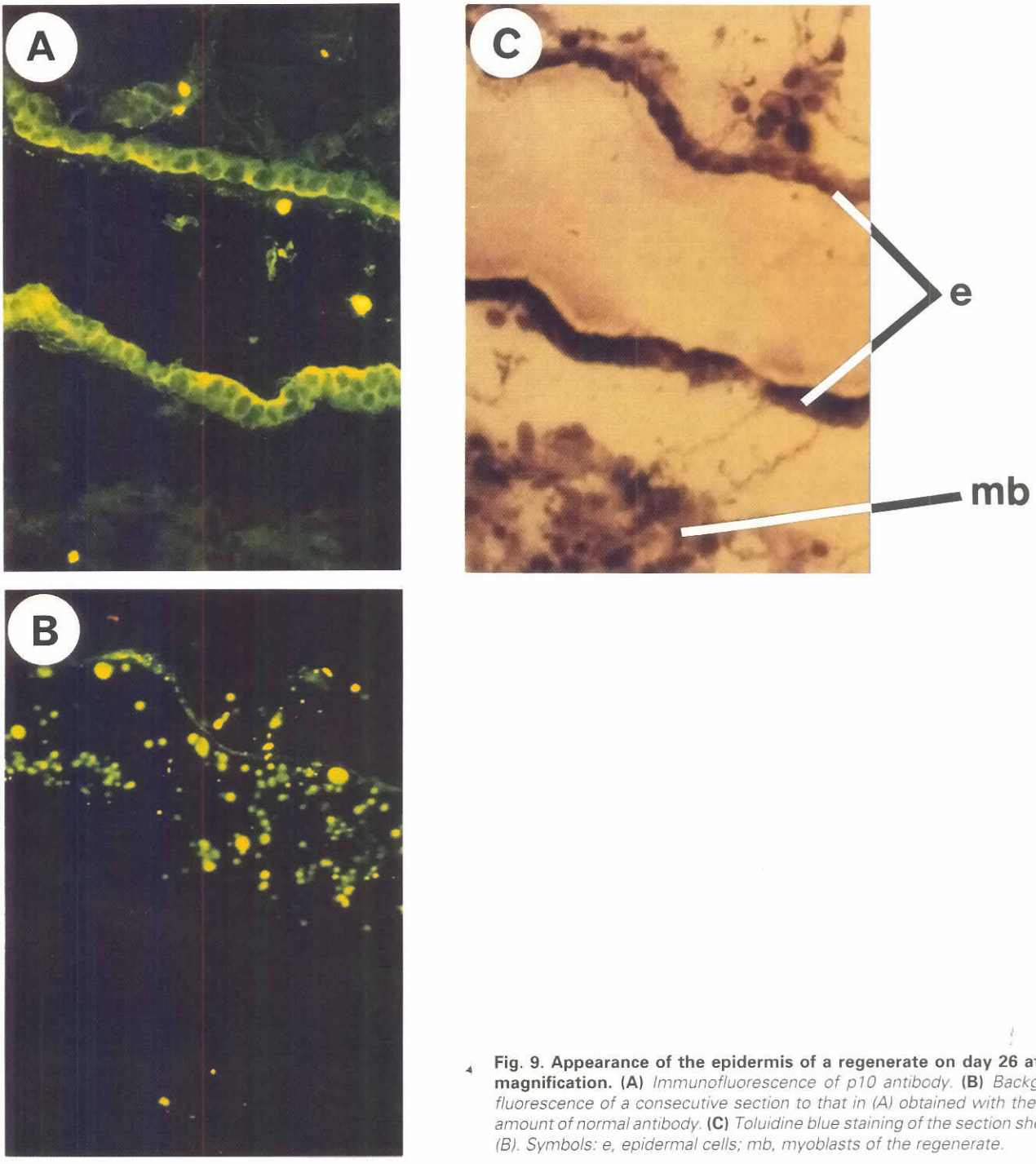

Fig. 9. Appearance of the epidermis of a regenerate on day 26 at high magnification. (A) *Immunofluorescence of p10 antibody.* **(B)** *Background fluorescence of a consecutive section to that in (A) obtained with the same amount of normal antibody.* **(C)** *Toluidine blue staining of the section shown in (B). Symbols: e, epidermal cells; mb, myoblasts of the regenerate.*

Affinity purification of antibody against p10

Antibody against p10 was raised by injecting 15 μg of the purified p10 into a male New Zealand white rabbit with complete Freund adjuvant, and giving two booster injections 14 and 24 days later. Affinity purification of the resulting antibody was performed with purified p10. For this, 100 μg of p10 was first electrophoresed in 12.5% polyacrylamide gel containing 0.2% SDS, and then the protein was blotted onto a polyvinylidene difluoride filter. The small region of the filter on which p10 was concentrated was excised and treated with skim milk solution. Then the strip of filter paper was incubated in 15 ml of 2.5-fold diluted antiserum in rinse solution at 4°C for 15 h with gentle shaking. The strip was rinsed well with rinse solution and cut into pieces, and the antibody specifically bound to p10 was extracted with 0.5 ml of 0.2 M glycine-HCl buffer, pH 2.8. The extract was neutralized with 1 M KOH, and a final concentration of 1% bovine serum albumin was added. Control IgG was purified from the preimmune serum by the method of McCauley and Racker (1973).

Immunofluorescence staining of the regenerates with antibody against p10

The regenerates of metathoracic legs were frozen in Tissue Tek and 10 μm sections were prepared on a gelatin-coated fluorescence-free glass slides. Samples were fixed in phosphate-buffered saline (10 mM phosphate buffer, pH 6.8, containing 130 mM NaCl, and 3 mM KCl) supplemented with 1.5% formaldehyde for 15 min, and washed with phosphate-buffered saline. Then the samples were incubated with 25 μl of affinity purified antibody solution for 1 h at room temperature. As negative controls, sequential sections were treated with control IgG. After washing well with buffered insect saline, the samples were incubated with 25 μl of FITC-conjugated swine anti-rabbit Ig for 30 min. The samples were kept in phosphate-buffered saline containing 2.5% (w/v) 1,4-diazabicyclo [2,2,2] octane (DABCO) for 5 min, and then immersed in 50% (v/v) fluorescence-free glycerol in phosphate-buffered saline containing 2.5% DABCO, and mounted on coverslips for examination.

The same samples used for the immunofluorescence studies were stained with Toluidine blue. For this, after removing the cover slips, the samples were treated in 1% (w/v) boric acid solution containing 0.5% (w/v) Toluidine blue for 1 min, and then washed well with water.

Acknowledgments

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