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PHENYLALANINE AMMONIA-LYASE FROM ILLUMINATED BUCKWHEAT HYPOCOTYLS AND PRODUCTION OF MONOCLONAL ANTIBODIES

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Fagopyrum esculentum*; Polygonaceae; buckwheat; phenylalanine ammonia-lyase; purification; monoclonal antibody; immunoblot.

Abstract—Phenylalanine ammonia-lyase (PAL) was purified to apparent homogeneity from illuminated buckwheat hypocotyls. The purification process involved ammonium sulphate fractionation, gel filtration, ion-exchange chromatography on DEAE-Bio-Gel, and chromatofocusing on PBE 94. The purified enzyme had a M_r of 330 000 ± 16 500 with a subunit size of M_r 85 000 ± 4000. Optimal activity of the enzyme was observed at pH 8.8. An apparent K_m value of 157 μM was determined for phenylalanine which was the only substrate utilized by the enzyme. Antibodies against the buckwheat PAL were developed using standard monoclonal techniques. A preparation containing antibodies from three monoclonal lines was able to precipitate 100% of the enzyme activity from crude buckwheat extracts. Western blots showed immunorecognition of proteins with M_r of 70 000; 65 000; and 55 000 in addition to the PAL subunit of M_r 85 000. These additional proteins appeared to be degradation products of PAL.

INTRODUCTION

Illuminated buckwheat hypocotyls have been used previously as model systems to study anthocyanin biosynthesis [1], channelling in metabolism [2], as a source of enzymes for antibody production [3], and for subcellular localization studies [4]. The advantages of this model system are that it produces relatively large amounts of anthocyanins [1, 2] and its photosynthetic activities are low. Hence, enzymes of the photosynthetic apparatus, that are present in leaves in large quantities, do not interfere significantly with the enzymes of secondary metabolism during purification.

L-Phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) is the key enzyme of the phenylpropanoid pathway, that channels phenylalanine from primary into secondary metabolism. Hence, its properties, subcellular localization, and genetic control are important factors in the general understanding of the metabolic processes that lead to the formation of aromatic compounds. We report here the isolation and characterization of PAL from illuminated buckwheat seedling and production of its monoclonal antibodies.

RESULTS AND DISCUSSION

Purification of PAL

The enzyme was purified from illuminated buckwheat seedlings using the conventional techniques of $(NH_4)_2SO_4$-precipitation, gel-filtration, ion-exchange chromatography and chromatofocusing. The majority of the PAL-activity was found in the protein fraction that precipitated between 40 and 60% (w/v) $(NH_4)_2SO_4$ saturation. This fraction, when subjected to gel-filtration, showed two peaks of activity (Fig. 1). A small peak was observed in the exclusion volume, that is most likely the membrane associated PAL [2]. The majority of the activity was recovered in later fractions, where the soluble enzyme was expected to elute. This PAL fraction was subjected to anion exchange chromatography on a DEAE-Biogel column. When the column was eluted with a linear K_2HPO_4–KH_2PO_4-gradient pH 8 from 50 to 200 mM concentration, two major activity peaks separated (Fig. 2). Since the concentration difference of the eluting buffer between the two PAL-peaks was small (100 and 120 mM respectively), the two peaks are thought to represent two different isoforms of PAL. The second peak that contained the majority of the PAL-activity had

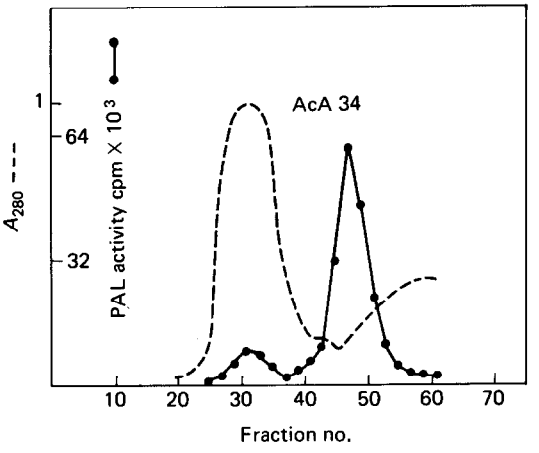

Fig. 1. Gel filtration of the 40–60% $(NH_4)_2SO_4$ precipitate on an Ultrogel AcA 34 column. Fractions containing the highest PAL activity were pooled for further purification.

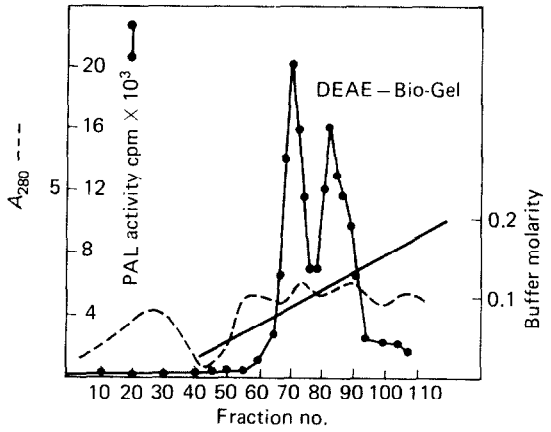

Fig. 2. Ion exchange chromatography on DEAE-BioGel of a PAL preparation after gel filtration. The sample was added in 20 mM K-Pi buffer and unabsorbed proteins washed off with two column volumes of the same buffer. Absorbed proteins were eluted with a linear K-Pi gradient between 50 and 200 mM concentration (solid line). Fractions 79–91 were combined and used for further purification of the enzyme.

a distinct shoulder on the higher buffer concentration side, suggesting the presence of other protein(s) with PAL activity, that carry different charges. This second peak possessed the majority of enzyme activity, and was subjected to chromatofocusing on a PBE94 column (Fig. 3). Development of the PBE column with the elution buffer separated further two PAL-activity peaks with the isoelectric points of 5.2 and 4.8, respectively, of which the second showed the presence of a distinct shoulder. This suggests the presence of three different isoforms of PAL that possess similar M_r, but differ in charges. Fractions with the highest activity of the second PAL-peak of the PBE94-column were pooled and analysed by SDS-PAGE. The electrophoretic analysis showed the presence of one protein band only in this preparation by both Coomassie Blue and silver staining. This preparation was used to characterize the enzyme and for the final screening of its monoclonal antibodies. The summarization of purification steps, specific activity of the preparations and yields is shown in Table 1.

The purification of PAL from the illuminated buckwheat hypocotyls posed many problems due to continuous degradation during the isolation process, which involved freezing and thawing of the preparations between the individual steps. This degradation was not prevented by the addition of phenylmethylsulphonylfluoride (PMSF), an inhibitor of serine proteinases commonly found in plants [5]. The enzyme showed major losses in both activity and protein content during the individual steps of the purification, particularly after freezing and thawing, resulting in low protein yields.

Properties of the isolated PAL

The properties of PAL from illuminated buckwheat hypocotyls that are shown in Table 2 closely resembled those reported from other higher plants [6]. An apparent M_r of 330 000 ± 16 500 was estimated by gel filtration using an Ultragel ACA34 column. SDS-PAGE gave a homogeneous subunit with an M_r of 85 000 ± 4000 suggesting a holoenzyme composition of four similar subunits. Purified PAL did not display negative cooperativity to L-phenylalanine as substrate, and gave a single apparent K_m of 157 μM. This value is similar to that reported for a preparation from illuminated mustard seedlings [7]. However, the enzyme from less pure preparations displayed negative cooperativity in substrate saturation kinetic experiments with values of K_m^{low} = 42 μm, and K_m^{high} = 220 μM. The purified enzyme preparation was active over a broad pH range, with optimal activity observed at around pH 8.8. During all stages of purification PAL utilized only phenylalanine as substrate.

Production and properties of monoclonal antibodies against PAL

Specific antibodies are necessary tools in subcellular localization studies. The low protein content of purified buckwheat PAL preparation, and the difficulties encoun-

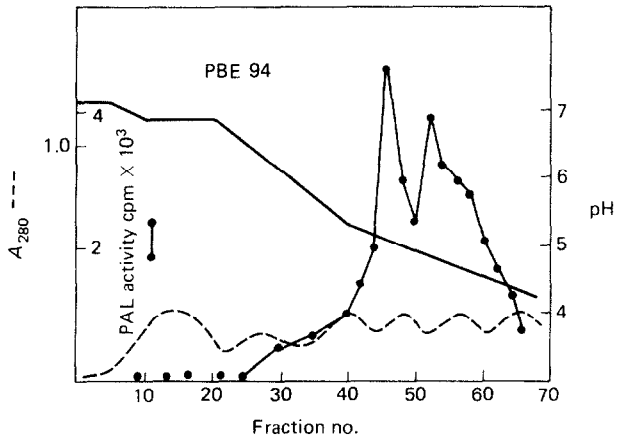

Fig. 3. Chromatofocusing of the PAL preparation after ion exchange chromatography on DEAE-BioGel. The column was developed with a linear pH gradient between pH 7 and 4, using Polybuffer 74. Fractions 50–57 were combined and used for enzyme characterization.

Table 1. Purification to apparent homogeneity and recovery of phenylalanine ammonia-lyase from 1280 g buckwheat hypocotyls

Fraction	Activity (μkat)	Protein (mg)	Spec. activity (μkat/g protein)	Relative purity	Recovery (%)
Homogenate	34.5	648.0	53	1	100
40–60% $(NH_4)_2SO_4$ Fraction	33.5	124.0	270	5	97
After AcA 34 Chromatography	17.0	19.1	895	17	49
After DEAE-BIOGEL Chromatography	5.4	2.1	2571	49	16
After PBE 94 Chromatofocusing	0.3	0.02	15000	282	1

Table 2. Properties of the phenylalanine ammonia-lyase isolated from buckwheat hypocotyls

pH-Optimum	8.8
M_r	330000 ± 16500
M_r Subunit	85000 ± 4000
K_m Phenylalanine	157 μM
V_{max}	47.7 nkat/l

tered in preventing degradation of the enzyme during the isolation procedure necessitated the production of monoclonal antibodies. We have used a PAL preparation purified through the DEAE-chromatography step for the immunization of Balb/cJ mice and monitored antibody production by indirect ELISA using the DEAE enzyme preparation as the antigen. Mouse No. 5 (Fig. 4) gave the highest antibody titre, and this was used for the establishment of hybridomas. The hybridoma colonies were screened first by indirect ELISA against the DEAE-enzyme preparation. Hybridomas that gave positive values were screened against an enzyme preparation that was purified to homogeneity. A total of seven hybridoma colonies were found to produce specific anti-PAL antibodies. We were successful in cloning four of these for antibody production.

The specificity of the anti-PAL antibodies was established by immunoprecipitation of enzyme activity from buckwheat hypocotyl homogenates, and also by immunoblotting. Incubation of an antibody preparation from cell culture supernatants with the buckwheat hypocotyl homogenates progressively decreased PAL activity in the supernatants (Fig. 5). Fifty per cent inhibition of PAL activity (1 pkat) of the buckwheat hypocotyl homogenate required the addition of 120 μl of the monoclonal cell culture supernatant containing the anti-PAL antibody. One hundred per cent inhibition was found upon addition of 250 μl of the cell culture supernatant. Controls containing pre-immune culture serum did not inhibit PAL activity. To further confirm the specificity of the anti-PAL antibody preparation we have separated crude tissue extracts and purified PAL preparations on SDS-PAGE-gels, electroblotted the protein to nitrocellulose membranes, and probed for PAL on the blots with the anti-PAL antibody, a rabbit anti mouse antibody preparation and a specific goat-anti rabbit IgG gold (20 nm) conjugate. We have observed faint immunostaining with the PAL subunits on the blots. However, the major protein bands that were recognized by the monoclonal antibody preparation had M_rs of 70000, 65000 and 55000 both in the crude tissue extracts from buckwheat hypocotyls, and from the purified PAL-preparations. Since both the purified PAL-preparation and the crude buckwheat hypocotyl extracts were frozen for storage, kept frozen at −40°, and thawed to room temperature immediately before use, the only feasible explanation for the above phenomenon seems to be the degradation of PAL upon freezing and thawing, or the presence of an PMSF insensitive endopeptidase with high catalytic activity, that was not detected by silver staining of the gels. The rapid loss of enzyme activity and protein upon freezing and thawing supports both possibilities. A similar phenomenon was reported during purification and polyclonal antibody production against PAL from French beans by Bolwell *et al.* [8].

EXPERIMENTAL

Materials. Biochemicals and immunochemicals were obtained from Sigma, radiochemicals from New England Nuclear, chromatographic supplies from LKB Instruments (AcA 34) Bio-Rad Laboratories (DEAE-Bio-Gel), or Pharmacia Fine Chemicals (PBE 94), electrophoretic supplies from Fisher, Pharmacia, or Hoeffer Scientific Instruments, Dulbecco's Modified Eagle's tissue culture medium and supplements were obtained from Sigma and Fetal Calf Serum from Gibco Laboratories. The goat anti-rabbit IgG gold (20 nm) conjugate was purchased from Boehringer Biochemicals. Buckwheat seedlings were grown as in ref. [2]. Balb/cJ mice were obtained from Jackson Laboratories, and the myleoma cell line was one maintained at the New York State Agricultural Experiment Station.

Purification of phenylalanine ammonia-lyase. All purification steps were performed at 4°. 1280 g of buckwheat (*Fagopyrum esculentum* M.) hypocotyls that had been illuminated for 18 hr were homogenized in four batches of 320 g each in 90 ml of 0.2 M Pi buffer containing 5 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulphonyfluoride, pH 8.8, in a Waring blender. 60 g insoluble polyvinylpolypyrrolidone was added, the homogenate stirred, and filtered under vacuum. The filtrates were fractionated by $(NH_4)_2SO_4$ pptn and the 40–60% (w/v) ppt. collected by centrifugation. The ppts were dissolved and desalted on an AcA 34 gel filtration column (750 × 25 mm), fractions were collected and assayed for PAL activity. Fractions containing the highest PAL activity were combined, concentrated in a Diaflow on column concentrator and stored frozen at

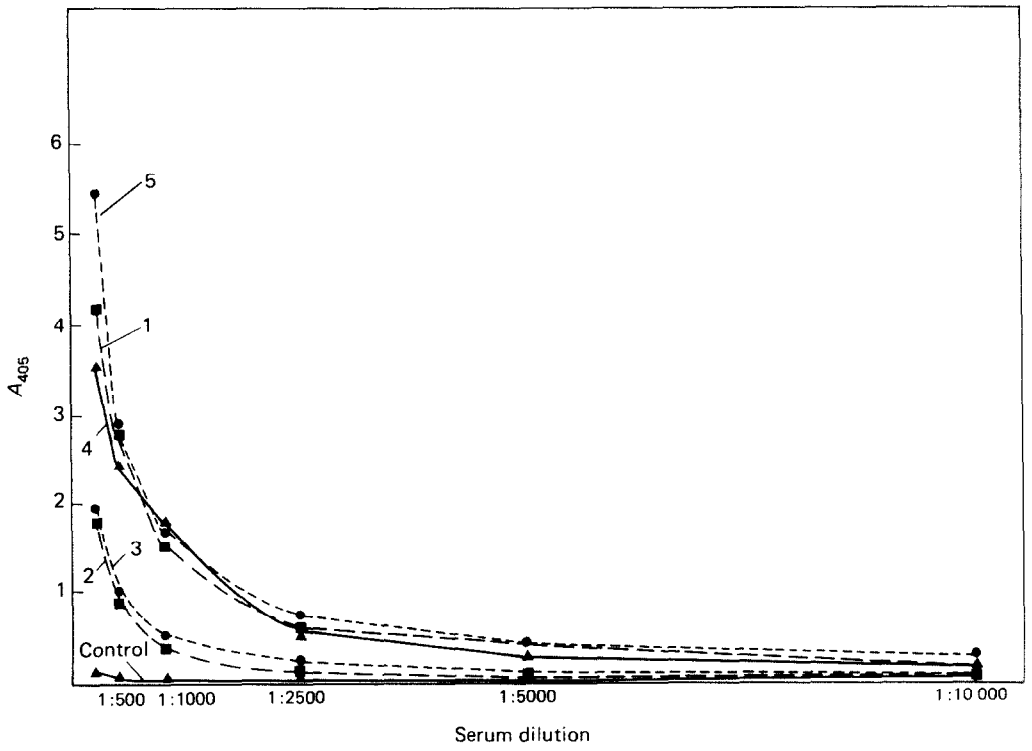

Fig. 4. Serum titres of Balb/cJ mice immunized with a PAL preparation purified through the DEAE-BioGel chromatography step, by indirect ELISA using Goat anti-mouse IgG alkaline phosphatase conjugate. Control serum was obtained from a non-immunized mouse.

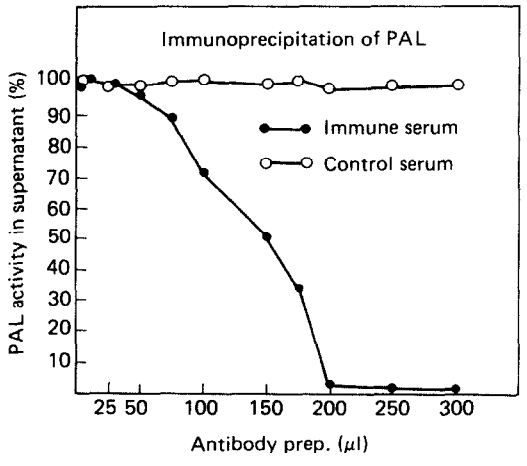

Fig. 5. Immunoprecipitation of PAL activity from crude buckwheat extracts. Increasing concentrations (1–300 μl) of antibody preparation incubated with 100 μl of extract for 1 hr at 37°, and for 16 hr at 4°, centrifuged at 11500 *g* for 15 min, and the supernatants assayed for PAL activity. Controls contained pre-immune mouse serum.

−40°. The frozen fractions from the four batches were pooled and diluted with deionized H_2O to a 10 mM buffer strength (5 mM 2-mercaptoethanol). This sample was applied to a DEAE-Bio-Gel column (170 × 25 mm) which was equilibrated and washed with 160 ml of 10 mM Pi buffer, pH 8.0 containing 5 mM 2-mercaptoethanol and then eluted with a linear gradient of 10–200 mM Pi buffer pH 8.0. Fractions were collected and assayed. Fractions 79–91, which contained the second peak of PAL activity were combined and concd. The buffer was exchanged to 20 mM imidazole-HCl pH 7.4 on a Bio-Gel P-10 column (50 × 15 mm) which was equilibrated with the same buffer. The sample was then applied to a Polybuffer exchanger (PBE94) column (170 × 15 mm) which was equilibrated with 20 mM imidazole–HCl buffer, pH 7.4. The column was developed with 420 ml Polybuffer 74. Fractions were collected and assayed. Fractions 50–57, corresponding to a IEP of 4.9, contained PAL which gave a homogeneous protein preparation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie Blue and silver staining. The molecular weight of PAL was determined by gel filtration on a calibrated AcA 34 column (750 × 25 mm), and by SDS-PAGE in 7.5% acrylamide gels. Protein was measured by the method of ref. [9].

Electrophoresis. SDS-PAGE was carried out in 7.5% gels according to ref. [10] with bovine serum albumin (BSA, M_r 68 000), ovalbumin (M_r 43 000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), and trypsinogen (M_r 24 000) as markers.

Enzyme assays. PAL activity was determined as follows: for monitoring during separations, 100 μl of the preparations were incubated for 10 min as described [2], and 100 μl aliquots of the EtOAc extracts were counted directly in a toluene cocktail (2.5 g PPO). In pH optimum and substrate specificity experiments saturating concentrations of substrate were used. The pH optimum was determined in 0.1 M K-Pi buffer between pH 5.0 and 9.0, and in 0.1 M glycine–NaOH between pH 9.0 and 10.0. Substrate specificity was determined with phenylalanine and tyrosine as substrates. In Michaelis–Menten kinetic experiments

the concentration of phenylalanine was varied between 1 μM and 1 mM. Cinnamic acid was confirmed as the product of the enzyme reaction by crystallization to constant specific radioactivity.

Monoclonal antibody production. Production of anti-phenylalanine ammonia-lyase antibodies was carried out by standard monoclonal techniques. One hundred micrograms of PAL protein, purified through DEAE-Bio-Gel chromatography, in 100 μl Pi buffered saline (PBS) was mixed with 100 μl of Freund's complete adjuvant and injected into each of five Balb/cJ mice. An additional 100 μg of PAL protein in Freund's incomplete adjuvant was injected four weeks later. Mice were bled one week later and the sera titres determined by indirect enzyme linked immunosorbent assay (ELISA). The mouse showing the highest serum titres was given an intraperitoneal injection of 50 μg PAL protein in sterile PBS three days prior to killing. The spleen was aseptically removed from the hyperimmune mouse and the cells fused with SP 2/0 myleoma cells as described by ref. [11].

Immunoprecipitation of enzyme activity. A plant extract was prepared from 25 g of buckwheat hypocotyls as described above. 100 μl aliquots of the extract were mixed with 0–300 μl of anti-PAL antibody preparation and 0.1 M Pi buffer pH 8.8 in a total volume of 400 μl. Reaction mixtures were incubated for 1 hr at 37° and 16 hr at 4°. The incubation mixtures were centrifuged for 15 min at 115 00 *g* at 4° and 100 μl aliquots of the supernatants assayed for PAL activity. Controls contained corresponding amounts of preimmune mouse serum.

Immunoblotting. Immunoblotting was carried out basically as described in ref. [12]. Samples were applied to SDS-PAGE (7.5%) gels and electrophoresed. Gels were blotted for 16 hr at 30 mA in a MRA electrophoretic blotting chamber. The nitrocellulose membrane was blocked at room temp. for 90 min with PBS containing 0.05% Triton X-100 and 0.5% Carnation non-fat dry milk (CNDM), and 4 hr with PBS containing 0.05% Triton X-100 and 4% CNDM. Nitrocellulose sheets were then incubated for 16 hr at room temp. with mouse anti-PAL antibody (1:50 dilution) in 10 ml PBS containing 0.05% Triton X-100, and 0.5% CNDM. The nitrocellulose sheet was washed four times for 15 min with 100 ml PBS containing 0.1% Triton X-100, and incubated with rabbit anti-mouse IgG (1:100 dilution) in 10 ml PBS containing 0.05% Triton X-100 and 0.5% CNDM for 4 hr. The nitrocellulose sheets were washed as above, and incubated with goat anti-rabbit IgG gold conjugate (1:100 dilution) in 10 ml PBS containing 0.5% Triton X-100 and 0.5% CNDM. PAL-antibody complexes appeared as red bands.

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REFERENCES

1. Amrhein, N. and Zenk, M. H. (1971) *Z. Pflanzenphysiol.* **64**, 145.
2. Hrazdina, G. and Wagner, G. J. (1985). *Arch. Biochem. Biophys.* **237**, 88.
3. Hrazdina, G., Lifson, E. and Weeden, N. F. (1986) *Arch. Biochem, Biophys.* **247**, 414.
4. Hrazdina, G., Zobel, A. M. and Hoch. H. C. (1987) *Proc. Natl Acad. Sci. U.S.A.* **84**, 8966.
5. Storey, R. D. (1986) in *Plant Proteolytic Enzymes* (Dalling, M. J., ed.) Vol. I, p. 119. CRC Press, Boca Raton.
6. Hanson, K. R. and Havir, E. A. (1981) in *The Biochemistry of Plants* (Conn, E. E., ed.) Vol. 7, p. 577. Academic Press, New York.
7. Gupta, S. and Acton, J. J. (1979) *Biochim. Biophys. Acta* **570**, 187.
8. Bolwell, G. P., Sap, J., Cramer, C. L., Lamb, C. J., Schuch, W. and Dixon, R. A. (1986) *Biochim. Biophys. Acta* **881**, 210.
9. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.
10. Laemmli, U. K. (1970) *Nature* **227**, 680.
11. deSt. Groth, F. and Scheidegger, D. (1980) *J. Immunol. Methods* **35**, 1.
12. Towbin, H., Staehelin, T. and Gordon, J. (1977) *Proc. Natl Acad. Sci. U.S.A.* **76**, 4350.