Journal of Chromatography, 455 (1988) 376–381
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 930

Note

Activity stain for polygalacturonase

THOMAS A. McKEON

U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, Albany, CA 94710 (U.S.A.)

(First received March 29th, 1988; revised manuscript received August 29th, 1988)

Polygalacturonase (E.C. 3.2.1.15) (PG) degrades the polygalacturonate component of pectin and is known to be involved in the breakdown of plant cell walls[1]. Hence, endogenous polygalacturonases influence fruit texture, which is an important quality component of harvested produce. With recent work suggesting roles for oligogalacturonides in plant development[2], the fact that these fragments are generated by the action of PG underlines the importance of detecting the presence of PG during stages of plant development.

As one part of a project to examine factors that influence the texture of ripening-delayed and lightly-processed (sliced) tomatoes we have developed an activity stain for polygalacturonase which provides detection of the activity after sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) and is thus based on separation by molecular size. This technique is useful in observing qualitative changes in PG levels and PG isoenzyme patterns during ripening of tomato fruit.

MATERIALS AND METHODS*

Pectinase purified from *Aspergillus niger* was purchased from Cappel-Worthington (Malvern, PA, U.S.A.). Acrylamide, bisacrylamide and SDS were purchased from Bio-Rad Labs. Polygalacturonic acid from orange was purchased from Sigma as the free acid, Sigma grade III. Molecular mass standards and Toluidine Blue O are also from Sigma. Tomato fruit (*Lycopersicon esculentum* Mill. cv. Rutgers) were harvested from plants grown in a greenhouse, graded based on internal (MG 4) or external coloring (as described in ref. 3), and pericarp was cut into pieces, frozen in liquid nitrogen and stored at −30°C. Polygalacturonase was extracted from the frozen pericarp as previously described[4], through the 75% ammonium sulfate precipitation and dialysis step. A unit of polygalacturonase activity generates 1 μmol of reducing sugar per minute from digestion of polygalacturonate. Reducing sugar produced by polygalacturonase action was measured using the 3,5-dinitrosalicylic acid method[5].

The procedure developed for polygalacturonase activity staining uses SDS-PAGE for separation based on molecular mass followed by digestion of endogenous substrate. The separating gel was 12.5% polyacrylamide, 2.7% cross-linked, and in

* Reference to a company or product named by the USDA is only for purposes of information and does not imply recommendation of the product to the exclusion of others.

addition to 0.375 *M* Tris–HCl, pH 8.8, 0.1% SDS[6], contained 0.67 mg/ml polygalacturonate (added as a 1% solution of polygalacturonic acid in water brought to pH 7 by addition of 1 *M* sodium hydroxide) and 100 μg/ml fibrinogen[7]. The stacking gel was 5% polyacrylamide, 2.7% cross-linked, containing 0.125 *M* Tris–HCl, pH 6.7, 0.1% SDS and contained no fibrinogen or polygalacturonate. Gels were run at room temperature with the Laemmli buffer system at 150 V through the stacking gel and 300 V through the separating gel. After electrophoresis, the dye front was marked and gels were shaken for 15 min with 250 ml of 0.01 *M* Tris–HCl, pH 8.3, in 20% isopropanol two times, twice for 15 min each in 250 ml of 0.01 *M* sodium acetate, pH 4.5 and finally incubated for 16 h at room temperature in 0.05 *M* sodium acetate, 0.20*M* sodium chloride, pH 4.5. After incubation, gels were washed briefly (30 min) with 0.01 *M* Tris–HCl, pH 8.3, stained for 5 min with 0.1% Toluidine Blue O in the same buffer and destained for approximately 1 h with three changes of Tris–HCl buffer. Use of 0.05% Ruthenium Red provides similar sensitivity to Toluidine Blue O, albeit with less intense background staining.

For quantitation, gels were scanned using a Bio-Med Model SL-TRFF densitometer. The light soure was a tungsten lamp with a 525-nm filter, and scans were carried out in the logarithmic range and inverse mode. The densitometer was equipped with an integrator which provided peak areas.

RESULTS AND DISCUSSION

The basis of the activity stain is the removal of SDS after electrophoresis, renaturation of enzyme in situ, digestion of substrate trapped in the gel matrix, removal of fragmented substrate from the gel by diffusion, and staining of the gel with Toluidine Blue O which binds to the polygalacturonate, leaving clear zones where PG has digested away the substrate. It is conceptually similar to the procedure developed by Blank *et al.*[8] for ribonuclease activity-staining. Fig. 1 illustrates the sensitivity of the staining technique, using purified *A. niger* pectinase. Lane a contains 50 ng of the purified protein. A clear, sharp band corresponding to a molecular mass of $57 \cdot 10^3$ appears in the gel. Lanes b and c contain 5 and 0.5 ng of the pectinase, and the band apparent in lane b demonstrates the detection of 5 ng of pectinase, corresponding to $8 \cdot 10^{-5}$ units of enzyme.

Fig. 2 is a comparison of detectable polygalacturonases present in tomato pericarp during ripening. Lanes a through d contain extracts from tomatoes of increasing ripeness. As previously reported[3,4,9] there is no detectable PG activity in the MG 4 stage (lane a) which represents the late mature green fruit. There is also no detectable PG in the turning stage (lane b) in agreement with levels of PG based on assay[3,4,9]. The pink (lane c) and ripe (lane d) samples illustrate the dramatic appearance and rise in PG activity during ripening and also give an indication of the isozyme distribution[9–11]. The high molecular mass PG 1 in lane d appears to be composed of two bands, the higher molecular mass of the two predominating. In the PG 2 pair the lower molecular mass band represents the major species. Previous workers have purified the PG 1 and PG 2[4,11–13] and demonstrated that they are immunologically related and, under stringent denaturing conditions (100°C, 2% SDS), PG 1 is converted to a subunit that is immunologically related and similar in size to PG 2 and a protein subunit that is not related to PG 2[10,12,13]. Relative molecular

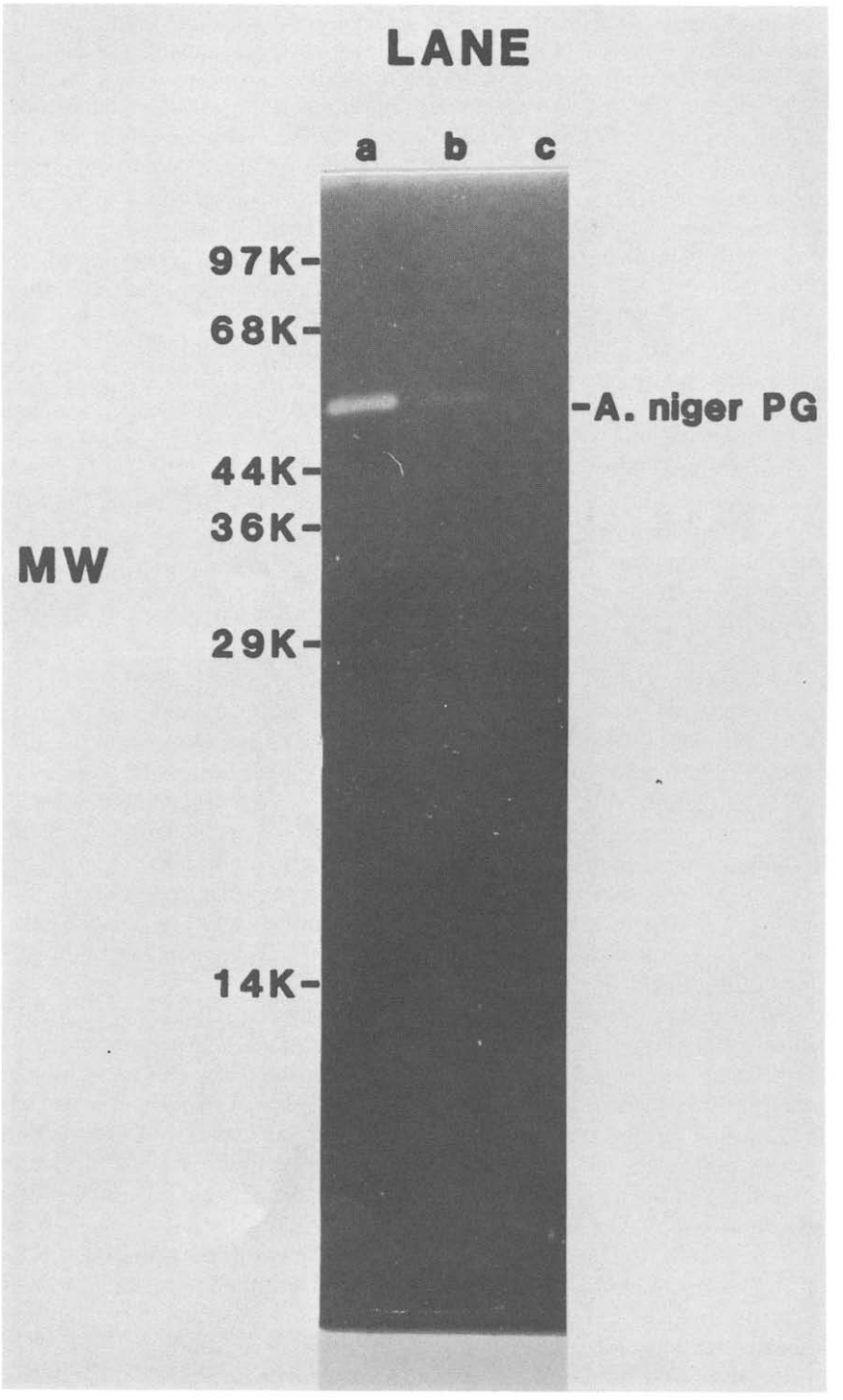

Fig. 1. Pectinase from *Aspergillus niger*. Samples were denatured in 2% SDS at 37°C and loaded on the gels. Loading buffer contained 0.02% fibrinogen to prevent losses due to surface binding. Each lane contained the specified number of units: (a) $8 \cdot 10^{-4}$; (b) $8 \cdot 10^{-5}$; (c) $8 \cdot 10^{-6}$.

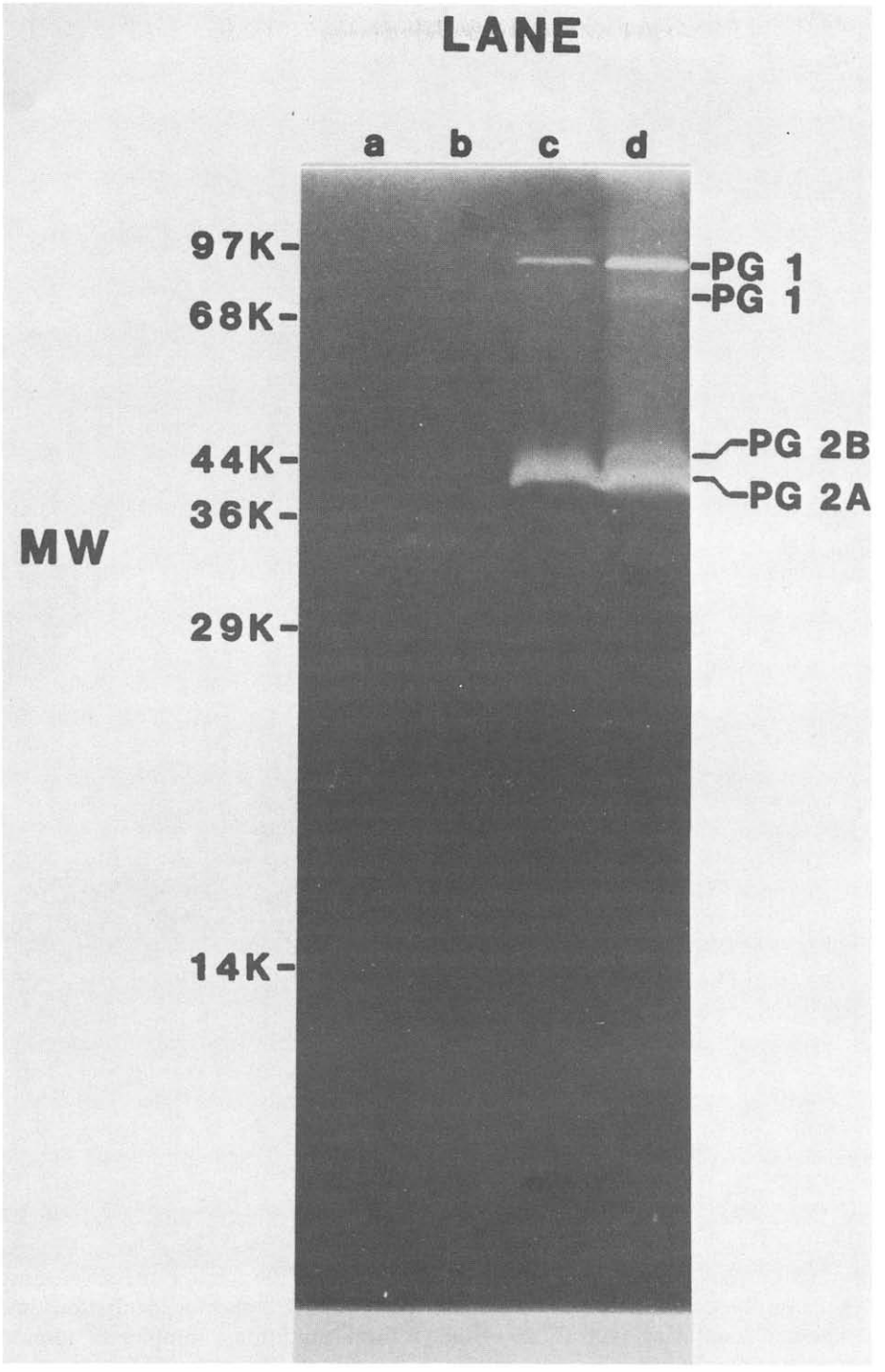

Fig. 2. Samples of tomato extracts were loaded on gels after denaturation at 37°C in 2% SDS. The developmental stage of the extracted tomato and the units loaded are: lane (a), MG4, no activity detected by assay; lane (b), turning, no activity detected by assay; lane (c), pink, 0.018 units; lane (d), ripe, 0.07 units.

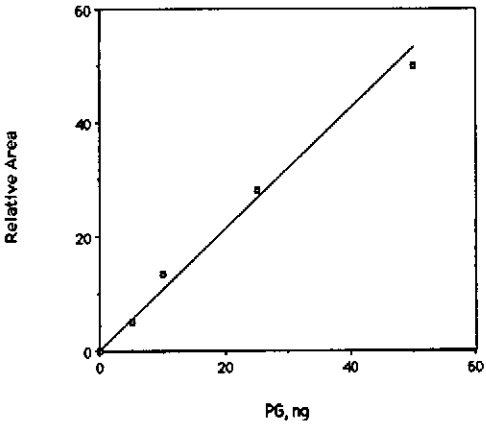

Fig. 3. A gel loaded with varying levels of *A. niger* pectinase was activity-stained after electrophoresis (gel not shown) and scanned as described in the Methods section. The units for relative peak area are directly proportional to absorbance and have been normalized to 50 for 50 ng pectinase.

masses reported for PG 1 include $84 \cdot 10^3$ (ref. 11), $100 \cdot 10^3$ (ref. 4), $115 \cdot 10^3$ (ref. 12) and $199 \cdot 1^3$ (ref. 13). The relative molecular masses found in the SDS, activity-stained gel are $89 \cdot 10^3$ and $79 \cdot 10^3$ (Fig. 2), and suggest, in accordance with the varying reported molecular weights for PG1, that PG1 exists in isoenzyme forms. The PG2 has been isolated and consists of two isoenzymes, PG2A and PG2B, with reported relative molecular masses of $43 \cdot 10^3$ and $46 \cdot 10^3$, respectively[12]). Molecular masses of $44 \cdot 10^3$ (ref. 11), $42 \cdot 10^3$ (ref. 4) and $47 \cdot 10^3$ (ref. 13) were reported for PG2 under conditions that do not resolve the two forms. The bands shown in Fig. 2, lanes c and d correspond to $43 \cdot 10^3$ and $47 \cdot 10^3$, indicating the resolution and detection of PG2A and PG2B by this activity stain.

Based on activity patterns shown in Figs. 1 and 2, the activity stain can be used for semi-quantitative purposes. As shown in Fig. 3, peak areas obtained from densitometry are linearly proportional to the amount of enzyme loaded over a ten-fold range from 5 to 50 ng. This linear relation of enzyme loaded to renatured activity indicates that the activity stain can reliably be used for quantitation of polygalacturonase isoenzymes.

There are three general types of activity-stain for polygalacturonase. The zymogram, which relies on diffusion of the enzyme into an overlay containing polygalacturonate followed by digestion and negative staining. The other types rely on the action of the enzyme *in situ* upon substrate incorporated prior to electrophoresis or diffused into the gel after electrophoresis[14]. Of two reported zymogram techniques[15], one uses non-denaturing conditions and separates on the basis of charge. However, the report[15] includes a zymogram technique used after SDS-PAGE. The overlay technique detected 0.1 units or more while the technique reported in this study detected $8 \cdot 10^{-5}$ units of enzyme activity. The reduced sensitivity of the zymogram for detecting polygalacturonase is due to incomplete enzyme diffusion, a shorter incubation time and harsher denaturing (100°C, 2% SDS, 2 min) conditions; samples of tomato polygalacturonase are not readily detected after denaturation at 100°C (data not

shown). The general disadvantage of overlay techniques is their reliance on diffusion which results in reduced sensitivity and loss of resolution of narrowly-resolved isoenzymes. One advantage of incorporating substrate in the gel is that a large, essentially non-diffusible substrate can be cast in the gel, providing a fixed uniform background after staining. Additionally, it has been suggested[16] that incorporated substrate may aid refolding of the enzyme to its native form after SDS denaturation.

Other activity stains incorporate polygalacturonate[17] or pectin[18] in the gel, both using non-denaturing conditions for sample treatment and electrophoresis. The advantage of the SDS activity stain is that separation is based on molecular mass. The use of a discontinuous gel buffer system provides stacking, resulting in a generally sharp banding pattern capable of resolving isoenzymes. These factors, coupled with the sensitivity of detection, make the SDS-PAGE activity stain for polygalacturonase a simple and effective means for detecting activity and for observing isoenzyme patterns during plant development.

ACKNOWLEDGEMENT

I would like to thank Ms. Rose de Lemos for typing this manuscript.

REFERENCES

1 J. M. Labavitch, *Ann. Rev. Plant Physiol.*, 32 (1981) 385–406.
2 M. McNeil, A. G. Darvill, S. C. Fry and P. Albersheim, *Ann. Rev. Biochem.*, 53 (1984) 625–663.
3 L. Y. Su, T. McKeon, D. Grierson, M. Cantwell and S F. Yang, *Hort. Science*, 19 (1984) 576–578.
4 G. A. Tucker, N. G. Robertson and D. Grierson, *Eur. J. Biochem.*, 112 (1980) 119–124.
5 W. W. Luchsinger and R. A. Cornesky, *Anal. Biochem.*, 4 (1962) 346–347.
6 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
7 A. Blank, J. R. Silber, M. P. Thelen and C. A. Dekker, *Anal. Biochem.*, 135 (1983) 423–430.
8 A. Blank, R. H. Sugiyama and C. A. Dekker, *Anal. Biochem.*, 120 (1982) 267–275.
9 C. J. Brady, G. MacAlpine, W. B. McGlasson and Y. Ueda, *Aust. J. Plant Physiol.*, 9 (1982) 171–178.
10 G. A. Tucker, N. G. Robertson and D. Grierson, *Eur. J. Biochem.*, 115 (1981) 87–90.
11 R. Pressey and J. K. Avants, *Biochim. Biophys. Acta*, 309 (1973) 363–369.
12 Z. M. Ali and C. J. Brady, *Aust. J. Plant Physiol.*, 9 (1982) 155–169.
13 M. Moshrefi and B. S. Luh, *Eur. J. Biochem.*, 135 (1983) 511–514.
14 N. Lisker and N. Retig, *J. Chromatogr.*, 96 (1974) 245 249.
15 J. L. Ried and A. Collmer, *Appl. Environ. Microbiol.*, 52 (1985) 305–310.
16 S. A. Lacks and S. S. Springhorn, *J. Biol. Chem.*, 255 (1980) 7467–7473.
17 H. Stegemann, *Hoppe-Seyler's Z. Physiol. Chem.*, 348 (1967) 951–952.
18 R. H. Cruickshank and G. C. Wade, *Anal. Biochem.*, 107 (1980) 177–181.