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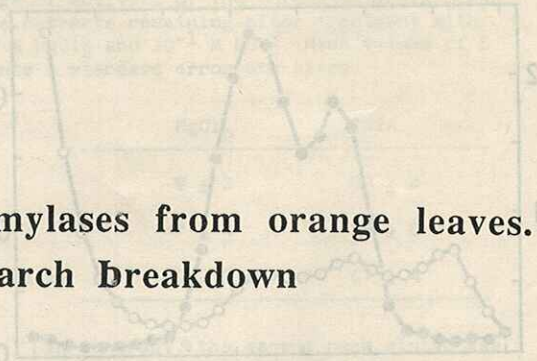

Amylases from orange leaves. Characterization and relation to starch breakdown

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ABSTRACT

Starch content in adult orange (Citrus sinensis L. Osbeck) leaves was highest at the end of the winter rest period and decreased during flowering and fruit set. Young inflorescence leaves accumulated starch until the June drop period to decrease to a low value at the end of it. Both α- and β-amylase activities were found in the leaves and the enzymes separated through Sephadex G100 filtration. By electrophoresis in polyacrylamide gels with 0.25 % amylopectine, α-amylase activity was resolved in 6 bands, and β-amylase in 7. Total amylolitic activity in the leaves was determined throughout the annual cycle in both adult and young leaves. Enzyme activities were not correlated with the rate of starch breakdown, so a regulatory rôle for α-amylase in starch mobilization in the leaves is not apparent.

INTRODUCTION.

Starch is the predominant reserve carbohydrate in Citrus (McCready, 1977), although the proportion of low molecular weight saccharides is high under low temperature conditions (Erickson, 1968; Yelenosky and Guy, 1977). A great proportion of the starch of the tree is stored in the leaves, in which it may represent up to 12 % of the total dry weight (Goldschmidt and Golomb, 1982). Starch content in leaves is maximum at the onset of the spring flush of growth and is depleted during flowering and fruit set, to increase again during the winter rest period (Jones and Steinacker, 1951; Lewis et al., 1964; Sanz et al., 1987). Although the pattern of starch mobilization is well known, the metabolic aspects have been insufficiently studied and further information about the enzymic pathways involved in starch degradation is needed.

The aim of this work is the characterization of the amylases present in Citrus leaves and the study of the relationship between enzyme activity and the changes in starch levels. Amylase activity is widespread in leaves (Gates and Simpson, 1968), and a concomitant action of amylases and phosphorylases in starch mobilization has been suggested (Okita et al., 1979; Steup and Schächtele, 1981). Amylase activity has already been reported in orange leaves (Argüelles and Guardiola, 1977) but neither its characteristics nor its relation to the variations in starch content has been elucidated.

MATERIALS AND METHODS

Plant material.- Leaf samples were taken from adult Washington navel orange trees (Citrus sinensis L. Osbeck) grafted on sour orange (Citrus aurantium L.). Two types of leaves were picked: leaves developed during the previous summer flush which were about 6 month old at the time of spring bud sprouting (old leaves), and leaves developed in inflorescences during the current spring flush (young leaves). Leaf samples were brought to the laboratory under refrigeration (0-4ºC) within 2 hours.

Enzyme extraction and amylase activity determination.- The leaves were homogenized with acid-washed sand in a mortar with prechilled sodium acetate buffer 0.1 M pH 5.0, in the presence of 1 mM $CaCl_2$. The crude protein extract was filtered and centrifuged at 10,000 g for 10 min. and used directly for the estimation of amylase activity.

Amylolitic activity in the extracts were determined using 0.07 % soluble starch as substrate, according to the method described by Monerri and Guardiola (1986a). Results are expressed as enzyme units per square centimeter of leaf lamina. A unit of enzyme reduces the absorbancy at 620 nm of the starch-iodine complex by 0.01 after 1 min of incubation under the assay conditions. For the selective inhibition of α- and β-amylases either 10^{-1} M ethylenediaminetetraacetic acid (EDTA) or 2 x 10^{-5} M $HgCl_2$ were added to the enzyme extracts 30 min prior to incubation with the substrate.

Enzyme characterization. For the separation of α- and β-amylase activities the protein of the enzyme extracts was precipitated with ammonium sulfate (20-100 % saturation). The precipitated protein was resuspended in a small volume of buffer and subjected to gel filtration using a 2.5 x 65 cm G100 Sephadex column. The peaks of activity were characterized as regards to pH, thermal stability by heating at 70 ºC for 15 min in the presence of 20 mM $CaCl_2$, and selective inhibitions of α- and β-amylase activities.

Slab gel electrophoresis of the crude protein extracts or the precipitated protein of the peaks obtained through gel filtration was performed as described by Ornstein (1964). Amylopectine azure at a final concentration of 0.25 % was added to the gel mixture before polymerization, and the amylase bands visualized by activity staining (Monerri and Guardiola, 1986b).

Starch analysis. The oven-dry powdered leaves were extracted exhaustively with boiling 80 % ethanol. Starch was then solubilized with 35 % perchloric acid and determined spectrophotometrically using the sulfuric acid-anthrone reagent (McCready et al., 1950).

RESULTS AND DISCUSSION

By gel filtration chromatography two peaks of amylase activity were partially separated (Figure 1). Their characteristics were determined excluding those fractions eluting at V_e/V_o between 1.65 and 1.85 to avoid the effects of overlapping. With this precaution, the peaks obtained were constituted essentially by different enzymes as assesed by their electrophoretic behaviour (Figure 2).

The first peak eluted presented characteristics reported as typical for β-amylase (Swain and Dekker, 1966; Jacobsen et al., 1970; Pech et al., 1971; Hildebrand and Hymowitz, 1981). The enzyme activity

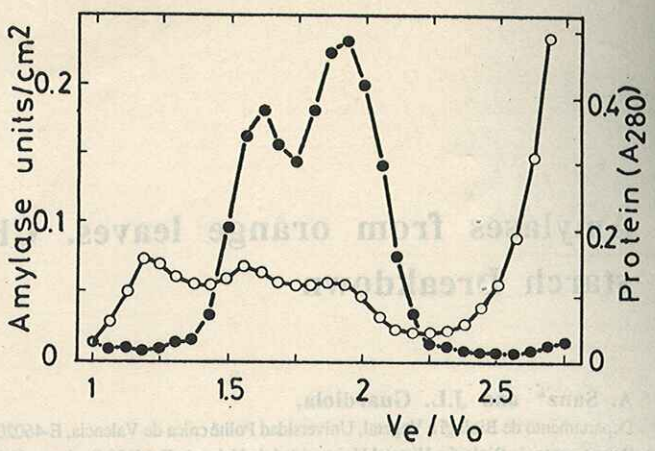

Figure 1.- Elution profile of the amylase activity (solid circles), and protein (open circles) from a column of Sephadex G100 (2.5 x 65 cm). Elution buffer 0.1 mM sodium acetate pH 5.0, 1 mM $CaCl_2$; flow, 0.58 ml/min; fraction volume, 7 ml.

associated to this fraction was drastically reduced in the presence of 2x10^{-5}M $HgCl_2$ while was unaffected by 10^{-1} M EDTA (Table 1). Enzyme activity showed a pH optimum of 5.0-6.0, and more than 75 % of the maximum activity remained at pH 3.0 (Figure 3), which is consistent with the low-pH resistance of β-amylases pointed out by several authors (Shain and Mayer, 1968; Tanaka et al., 1970). Further, this fraction was unable to degrade completely the amylopectine molecule, and in the slab gels the bands of activity showed a faint pink colour due to the β-limit dextrins (Doehlert and Duke, 1983).

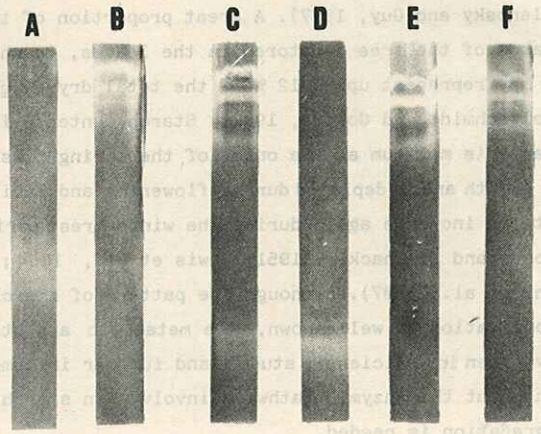

Figure 2.- Zymogram patterns for the first (A) and the second (B) Sephadex chromatography peaks, and for unpurified leaf protein extracts (C-F). Crude protein extracts untreated (C), treated with 10^{-1} M EDTA (D) or 2 x 10^{-5} M $HgCl_2$ (E), and heated 15 min at 70 ºC (F).

TABLE 1

Percentage of activity of the chromatographic peaks and crude extracts remaining after treatment with 2×10^{-5} M $HgCl_2$ and 10^{-1} M EDTA. Mean values of 5 experiments ± standard error are given.

	$HgCl_2$	EDTA
Peak 1	9 ± 3	90 ± 4
Peak 2	47 ± 4	23 ± 8
Crude extracts	89 ± 2	67 ± 4

In contrast, the second peak eluted was capable to degrade completely the amylopectine molecule to iodine-unreactive compounds, was heat stable at 70 ºC for 15 min in the presence of 20 mM Ca^{+2} ions (Figure 2) and was strongly inactivated by EDTA and by low pH (Figure 3). Its partial inhibition by $HgCl_2$ is higher than generally admitted for α-amylases (Pech et al., 1971), but endoamylases sensitive to sulfhydril reagents have been reported both in leaves (Okita et al., 1979; Okita and Preiss, 1980) and in germinating seeds (Doehlert and Duke, 1983).

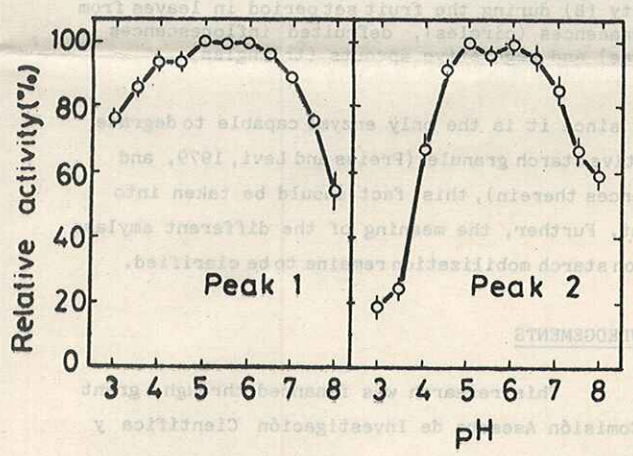

Figure 3.- Relative activity of the enzymic peaks as a function of pH. Each curve represents the mean value of 3 (peak 1) or 2 (peak 2) separate determinations with each of the buffers tested (0.25 M sodium acetate, 0.25 M tris-maleate, 0.01 M citrate-0.02 M phosphate).

A total of 13 bands of amylase activity were separated by gel electrophoresis of unpurified protein precipitates from adult leaves (Figure 4). From their response to the selective inhibitors (Figure 2) bands 1 to 6, which elute in the second peak in gel filtration can be classified as α-amylases. Bands 7 to 13, which elute in the first peak, represent characteristics of β-amylases. These bands are difficult to demonstrate simultaneously in the same gel since the conditions of incubation needed to show the faintest ones (bands 7,8 and 9) cause an excessive spreading of the most intense ones, which overlap (bands 2, 3, 5, 12 and 13). The development of this electrophoretic pattern is related to leaf development. In 4-month old leaves band 5 is absent, and bands 10 and 11 have an intensity similar to bands 12 and 13 as judged by gel staining (Figure 4). One month latter, band 5 is distinctly present while the intensity of bands 12 and 13 is higher than for bands 10 and 11, a characteristic invariably found in older leaves extracts. The relative intensity of the different bands in extracts from older leaves was rather constant despite the wide variations in total amylase activity (see below).

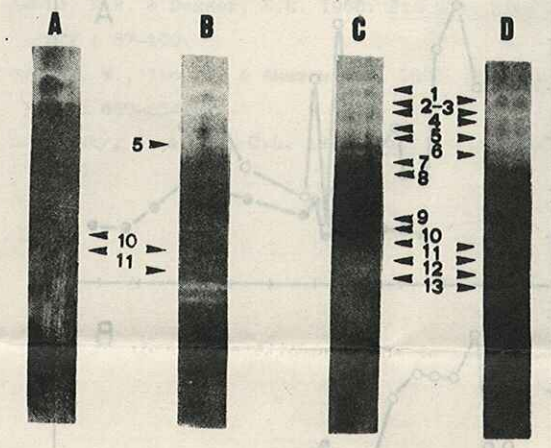

Figure 4.- Changes in the zymogram patterns during leaf development. Zymograms from 4-month old (A), 5-month old (B) and adult (8-month old, C-D) leaves. For adult leaves enzyme activity applied was 5.3 (C) and 2.2 (D) units.

The presence of multiple α-amylase bands has been described as the result of molecular rearrangements of the same enzyme molecules (Nádudvari-Márkus et al., 1982). The possibility that such a rearrangement may occur in our extracts during extraction and purification may be dismissed from the differences in the pattern of bands associated with leaf development.

Despite the sensitivity of the partially purified enzyme extracts to the selective amylase inhibitors, these compounds were ineffective to inhibit amylase activity in crude leaf protein extracts (Table 1), a difficulty already encountered with other plant extracts (Monerri and Guardiola, 1986a). Therefore, total amylolitic activity (α+β) was determined and related to starch content in leaves. Figure 5 shows the changes in old and young inflorescence leaves from bud sprouting until fruit ripening. No clear relation-

ship is apparent between amylase activity and starch degradation and / or content in the leaves. In old leaves, the maximum in starch level was found at the end of the winter rest and the rate in starch depletion is not related to enzyme activity, which decreases during May when starch is lost at a maximum rate. Parallel changes in starch content and amylase activity in young inflorescence leaves are apparent as well during June, coinciding with the drop of developing fruitlets. In both old and young leaves, amylase activity during June varied in the same way suggesting a common regulatory mechanism, but no relation to starch content appears.

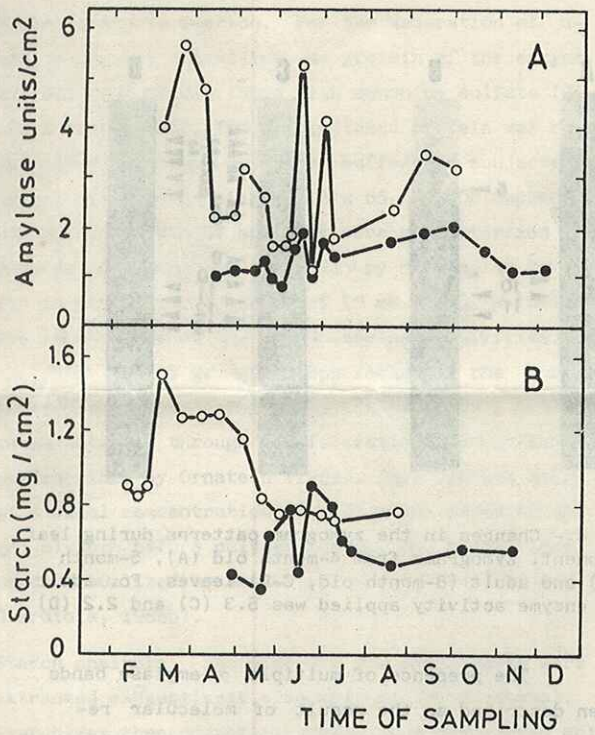

Figure 5.- Seasonal changes of the total amylase activity (A) and starch content (B) in young (solid circles) and old leaves (open circles).

A better relationship between amylase activity and leaf starch was found during transient changes. Excising the developing fruitlets during the June drop period causes a reduction in leaf starch content within nine days to the levels found in leaves from vegetative sprouts (Figure 6), an effect previously reported (Sanz et al., 1987). This starch loss is preceded by an increase in amylolitic activity which may indicate a regulatory rôle of this enzyme in starch mobilization.

However, in all cases, enzyme activity as measured "in vitro" is much higher than necessary to account for the rate of starch loss; it is probable that inactive enzymes "in vivo" are extracted giving activities by far higher than the real ones inside the cells, and better relationships with starch breakdown are obtained when relative changes in activity are used instead absolute values. To ascertain if α-amylases play a regulatory rôle in starch breakdown as it is

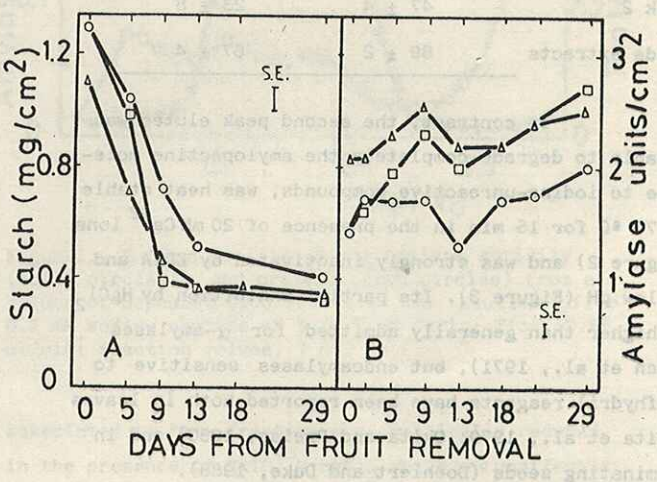

Figure 6.- Changes of starch content (A) and amylase activity (B) during the fruit set period in leaves from inflorescences (circles), defruited inflorescences (squares) and vegetative sprouts (triangles).

likely since it is the only enzyme capable to degrade the native starch granule (Preiss and Levi, 1979, and references therein), this fact should be taken into account. Further, the meaning of the different amylase bands on starch mobilization remains to be clarified.

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REFERENCES

Argüelles, T. & Guardiola, J.L. 1977. J. Hort. Sci., 52 : 199-204.

Doehlert, D.C. & Duke, S.H. 1983. Plant Physiol., 71: 229-234.

Erickson, L.C. 1968. In: Reuther, W., Batchelor, L.D. & Webber, H.J. (Eds). The Citrus Industry, Vol 2 University of California. pp 86-122.

Gates, J.W. & Simpson, G.M. 1968. Can. J. Bot., 46 : 1459-1462.

Goldschmidt, E.E. & Golomb, A. 1982. J. Amer. Soc. Hort. Sci., 107 : 206-208.

Hildebrand, D.F. & Hymowitz, T. 1981. Physiol. Plant.,

53 : 429-434.

Jacobsen, J.V., Scandalios, J.G. & Varner, J.E. 1970. Plant Physiol., 45 : 367-371.

Jones, W.W. & Steinacker, M.L. 1951. Proc. Amer. Soc. Hort. Sci., 58 : 1-4.

Lewis, L.N., Coggins, C.W. & Hield, H.Z. 1964. Proc. Amer. Soc. Hort. Sci., 84 : 147-151.

McCready, R.M. 1977. In: Nagy, S., Shaw, P.E. & Veldhuis, M.K. (Eds). Citrus science and technology. Vol 1. The Avi Publishing Co., Inc. Wesport, Conneticut. pp 74-109.

McCready, R.M., Silveira, V. & Owens, H.S. 1950. Anal. Chem., 22 : 1156-1158.

Monerri, C. & Guardiola, J.L. 1986a. Rev. Agroquím. Tecnol. Aliment., 26 : 219-233.

Monerri, C. & Guardiola, J.L. 1986b. Rev. Agroquím. Tecnol. Aliment., 26 : 424-434.

Nádudvari-Márkus, V., Párkány-Gyárfás, A. & Vámos-Vigyázó, L. 1982. J. Chrom., 241 : 229-235.

Okita, T.W., Greenberg, E., Kuhn, D.N. & Preiss, J. 1979. Plant Physiol., 64 : 187-192.

Okita, T.W. & Preiss, J. 1980. Plant Physiol., 66: 870-876.

Ornstein, L. 1964. Ann. N. Y. Acad. Sci., 121 : 321-343.

Pech, J.C., Bonneau, G. & Fallot, J. 1971. C. R. Acad. Sci., 273 : 775-778.

Preiss, J. & Levi, C. 1979. In: Gibbs, M. & Latzko, E. (Eds). Encyclopedia of Plant Physiol. Vol 6. Springer Verlag. Berlin-Heildelberg-New York. pp 282-312.

Sanz, A., Monerri, C., González-Ferrer, J. & Guardiola, J.L. 1987. Physiol. Plant., 69 : 93-98.

Shain, Y. & Mayer, A.M. 1968. Physiol. Plant., 21 : 765-776.

Steup, M. & Schächtele, C. 1981. Planta, 153 : 351-361.

Swain, R.R. & Dekker, E.E. 1966. Biochim. Biophys.Acta, 122 : 87-100.

Tanaka, Y., Ito, T. & Akazawa, T. 1970. Plant Physiol., 46 : 650-654.

Yelenosky, G. & Guy, C.L. 1977. Bot. Gaz., 138: 13-17.