Calcium regulation of the secretion of α-amylase isoenzymes and other proteins from barley aleurone layers

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Abstract. The effect of calcium on the secretion of α-amylase (EC 3.2.1.1) and other hydrolases from aleurone layers of barley (Hordeum vulgare L. cv. Himalaya) was studied. Withdrawal of Ca\(^{2+}\) from the incubation medium of aleurone layers preincubated in 5 μM gibberellic acid (GA\(_3\)) and 5 mM CaCl\(_2\) results in a 70–80% reduction in the secretion of α-amylase activity to the incubation medium. Agar-gel electrophoresis shows that the reduction in α-amylase activity following Ca\(^{2+}\) withdrawal is correlated with the disappearance of group B isoenzymes from the incubation medium. The secretion of isoenzymes of group A is unaffected by Ca\(^{2+}\). The addition of Ca\(^{2+}\) stimulates the secretion of group-B isoenzymes but has no measurable effect on either the α-amylase activity or the isoenzyme pattern of aleurone-layer extracts. Pulse-labelling experiments with \(^{35}S\)methionine show that Ca\(^{2+}\) withdrawal results in a reduction in the secretion of labelled polypeptides into the incubation medium. Immunochemical studies also show that, in the absence of Ca\(^{2+}\), α-amylase isoenzymes of group B are not secreted into the incubation medium. In addition to its effect on α-amylase, Ca\(^{2+}\) influences the secretion of other proteins including several acid hydrolases. The secretion of these other proteins shows the same dependence on Ca\(^{2+}\) concentration as does that of α-amylase. Other cations can promote the secretion of α-amylase to less and varying extents. Strontium is 85% as effective as Ca\(^{2+}\) while Ba\(^{2+}\) is only 10% as effective. We conclude that Ca\(^{2+}\) regulates the secretion of enzymes and other proteins from the aleurone layer of barley.

Key words: Aleurone – α-Amylase – Calcium and enzyme secretion – Enzyme secretion – Gibberellin – Hordeum (enzyme secretion).

Introduction

The importance of calcium for the production of α-amylase by the barley aleurone layer was established by Chrispeels and Varner (1967). These workers showed that while gibberellic acid (GA\(_3\)) alone was sufficient to promote α-amylase production by de-embryonated half-seeds of barley, Ca\(^{2+}\) in addition to GA\(_3\) was necessary to obtain a high rate of α-amylase production in aleurone layers removed from the starchy endosperm. Chrispeels and Varner proposed that the absence of Ca\(^{2+}\) secreted α-amylase was irreversibly inactivated. They supported this hypothesis by showing that the activity of purified malt α-amylase was nearly abolished when added to incubation media containing barley aleurone layers but no Ca\(^{2+}\) (Chrispeels and Varner 1967). Subsequently, it was shown that the effect of Ca\(^{2+}\) on α-amylase production by barley aleurone was selective. Jacobsen et al. (1970) showed that the additional α-amylase activity appearing when Ca\(^{2+}\) was added to the incubation medium of isolated aleurone layers was largely the consequence of the appearance of two new isoenzymes (group-B α-amylases; Jacobsen and Knox 1973). Because α-amylase is a Ca\(^{2+}\)-containing metalloenzyme (see review by Fisher and Stein 1960), and because the group-B isoenzymes were sensitive to ethylenediaminetetraacetic acid (EDTA), it appeared that Ca\(^{2+}\) was required for the stability of group B α-amylases.

Calcium has also been implicated in the process of α-amylase secretion from the barley aleurone
layer by Varner and Mense (1972) and Moll and Jones (1982). Varner and Mense argued that the process of enzyme secretion could be separated into two distinct steps, transport of enzyme across the plasma membrane, which they called secretion, and movement of the enzyme through the matrix of the aleurone cell wall, which they defined as enzyme release. They presented evidence showing that α-amylase release was regulated by cations, especially Ca\(^{2+}\), Mg\(^{2+}\), and K\(^{+}\). Varner and Mense (1972) proposed that the aleurone cell wall acted as an ion-exchange material and that the role of cations was to facilitate the movement of the enzyme through the wall. More recently, Moll and Jones (1982) studied the kinetics of α-amylase production by single aleurone layers under various conditions and proposed that the effect of Ca\(^{2+}\) in regulating the secretion of α-amylase was exerted primarily not at the cell wall but at the plasma membrane.

It has been well established that Ca\(^{2+}\) plays a key role in the regulation of secretion in animal tissues (for reviews and surveys, see Rubin 1974; Foreman et al. 1976; Matthews 1979). These effects of Ca\(^{2+}\) include the control of the secretion of α-amylase and other hydrolases from exocrine glands, the secretion of hormones from endocrine glands, the secretion of neurotransmitters, and the movement of ions across cell membranes.

The present work was designed to define the role of Ca\(^{2+}\) in the production of α-amylase and other proteins by isolated barley aleurone layers more closely. The experiments reported in this paper do not permit us to distinguish between plasma membrane and cell-wall phenomena in controlling enzyme secretion, rather we consider enzyme secretion to include all processes involved in moving enzymes out of the cell into the incubation medium. Our results show that Ca\(^{2+}\) exerts a selective effect on the secretion of α-amylase isoenzymes. The secretion of two isoenzymes is shown to depend on the presence of Ca\(^{2+}\), while the secretion of two other α-amylase isoenzymes is independent of Ca\(^{2+}\). In addition, Ca\(^{2+}\) strongly influences the secretion of other acid hydrolases from the aleurone layer.

Material and methods

**Plant material.** Caryopses of barley (Hordeum vulgare L. cv. Himalaya, 1979 harvest; Department of Agronomy, Washington State University, Pullman, USA) were prepared for imbibition as described by Chrispeels and Varner (1967). Deembryonated half-seeds were imbibed on sterile filter paper in sterile H\(_2\)O for 4 d at 20 °C (Jones and Jacobsen 1982), and the aleurone layers were removed from the starchy endosperm as described in Chrispeels and Varner (1967).

**Incubation.** The isolated aleurone layers were washed briefly in sterile H\(_2\)O and incubated in 2–2.5 ml of a medium containing 1–10 μM gibberellic acid (GA\(_3\); potassium salt, Calbiochem, San Diego, CA, USA), 2.5–10 mM CaCl\(_2\), and 30 μM chloramphenicol (Sigma Chemical Co., St. Louis, Mo., USA) for 20–24 h at 25 °C, prior to incubation in GA\(_3\) (1–10 μM) in the presence or absence of CaCl\(_2\) or other cations at 25 °C.

**Radiolabelling.** Following incubation in GA\(_3\) (5 μM) and CaCl\(_2\) (10 mM) for 21 h, the aleurone layers were rinsed twice with sterile, distilled H\(_2\)O the incubated in new medium containing GA\(_3\) (5 μM), CaCl\(_2\) (10 mM) and 0.63-10\(^{-11}\) Bq ml\(^{-1}\) \([^{35}\text{S}]\)methionine (3.7·10\(^{-12}\) Bq mol\(^{-1}\); Radiochemical Centre, Amersham, UK) for 2 h. After labelling, the aleurone layers were washed for 2 min in 10 mM CaCl\(_2\) and 1 mM methionine and for 5 min in H\(_2\)O, then transferred to new medium containing GA\(_3\) (5 μM) with or without 10 or 20 mM CaCl\(_2\). Samples of the incubation medium (25 μl) were removed at 20-min intervals and mixed with 4.2 volumes of ice-cold 95% ethanol. Ethanol-insoluble radioactivity was determined using the filter-disc method of Mans and Novelli (1961).

**Tissue homogenization.** For recovery of intact organelles, tissues was homogenized with razor blades according to the procedure of Jones (1980). After centrifugation at 1,080 g for 10 min (Sorvall SS34 rotor; Du Pont Instruments, Newtown, Conn., USA), the supernatant was layered over a 1.5-cm diameter, 18-cm long Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, N.J., USA) column (Firm 1975) and eluted with homogenizing buffer. Fractions (1 ml) were collected automatically until the column was completely eluted (about 20 ml). The column fractions were monitored for ultraviolet absorbance at 280 nm and for α-amylase activity. The turbid, particulate fractions were pooled and designated the organelle fraction, and the fractions containing soluble enzyme activity were pooled and designated the soluble fraction (Firm 1975; Jones 1980). When fractionation of the homogenate was not desired, aleurone layers were ground in a glass homogenizer in 10 mM CaCl\(_2\) (3 ml per 10 aleurone layers). The homogenate was centrifuged at 1,080 g for 10 min and the pellet discarded.

**Agar-gel electrophoresis.** Agar-gel electrophoresis was carried out according to the procedures of Jacobsen et al. (1970) except that the running buffer was 4 mM K\(_2\)HPO\(_4\) (pH 8.8) and the agar was also made up in this buffer. Amylase was visualized using the starch-iodine procedure of Jacobsen et al. (1970).

**Sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE)** was performed either on gradient (12.5–20%) polyacrylamide gels as described in detail by Spencer et al. (1980), or on discontinuous 12.5% polyacrylamide gels as described by Laemmli (1970). Protein bands on gels were visualized by staining with Coomassie brilliant blue R 250 or with the silver method of Merril et al. (1981). Radioactivity was detected by fluorography as described by Jones and Jacobsen (1982).

**Enzyme and protein assays.** α-Amylase (EC 3.2.1.1) was assayed using the starch-iodine method described by Jones and Varner (1967), and acid phosphatase (p-nitrophenyl phosphatase, EC 3.1.3.2) was assayed as described by Ray et al. (1969), β-D-glucanase (EC 3.2.1.39) was determined as described by Taiz and Jones (1970), protease using Azocoll (Sigma Chemical Col. as described by Chrispeels and Boulter (1973), and ribonuclease as described by Wilson (1963). Protein was determined using the method of Lowry et al. (1951).