Phytochemistry, 1977, Vol. 16, pp. 1491–1494. Pergamon Press. Printed in England.

ISOENZYMES OF α-AMYLASE DURING POD DEVELOPMENT OF FIELD BEANS*

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(*Revised received* 9 *April* 1977)

Key Word Index—*Vicia faba*; Leguminosae; field bean; pod; α-amylase; isoenzyme; phosphorylase; polyacrylamide gel electrophoresis; ontogenesis.

Abstract—The pod mesophyll of field beans accumulates large amounts of starch during stage 1 of embryogenesis, which is later utilized during stage 2. The activity of starch degradation in the pod is under metabolic control of the enclosed seeds. Changes in the isoenzyme pattern of α–amylase and not starch phosphorylase coincide with the beginning of the starch degradation period in the pods. Mesophyll cells of the pods contain the same α-amylase isoenzymes as the endocarp but exhibit a higher α-amylase activity that parallels the much higher starch content of this tissue in comparison to the endocarp. Regulation of starch breakdown may be mediated at least in part by the formation of a special α-amylase isoenzyme.

INTRODUCTION

The cells of the pod mesophyll of field bean fruits accumulate large amounts of starch during stage 1 of embryogenesis. The subsequent period of starch degradation coincides with stage 2 of embryogenesis, when seeds are growing rapidly and synthesize and accumulate large quantities of starch and protein [1]. In previous work it was demonstrated that starch breakdown in the pod mesophyll is under the control of the seed since removal of the seeds reduces starch degradation in the pod. Starch breakdown can also be increased by exposing whole fruits to a shortage of sucrose. The increased starch reactivation is paralleled by a corresponding increase in amylolytic enzyme activity [2]. The rise in amylolytic enzyme activity may result either from induced catalytic activity of enzyme proteins that were already present in the cells previously in an inactive state or from *de novo* biosynthesis of enzyme protein. The development of new isoenzyme patterns at this stage is investigated in the present paper.

RESULTS

Phosphorylase and amylase isoenzymes in full grown pods

Electrophoretic separation of soluble proteins from field bean pods 5 weeks after anthesis and subsequent incubation of the starch containing polyacrylamide gels with phosphate buffer gave 9 zones without blue iodine staining. At least 4 of these zones correspond to native startch degrading enzymes. One bright zone was always located at the buffer front and could be eliminated by dialysing the extract for 15 hr against extraction buffer or by ammonium sulphate precipitation with subsequent dialysis. Some reducing low MW compounds may cause the unspecific reaction at the buffer front. A rapidly disappearing yellowish zone frequently was located between the 2 phosphorlyase positions at low R_p-values. The 3 weak amylolytic zones at R_p 0.54, 0.57 and 0.61 (Fig. 1c), respectively, probably are association products of the main amylases (Fig. 1b, c). These occupy positions very close together at R_p 0.72 and 0.74.

After combined isolation of the amlylases at R_p 0.72 and 0.74 by preparative gel electrophoresis the presence of the enzyme was always checked by zymography on starch containing analytical gels. In addition to the main amylase double zone at R_p 0.72 and 0.74 at least the strongest of the 3 weak amylolytic zones at R_p 0.54, 0.57, and 0.61, respectively, reappeared from R_p 0.72 and 0.74 (Fig. 2b, c). Comparison of gel a with gel b of Fig. 1 indicates the coincidence of the two amylolytic zones with the corresponding protein zones. Incubation of 0.01% starch containing gels with glucose–1–phosphate in otherwise phosphate free buffer led to the conclusion that the two slowly moving starch degradation zones near the top of the gels at R_p 0.07 and 0.20 (Fig. 1d) correspond to starch phosphorylase, whereas 5 other starch degradation zones represent amylase activities (Fig. 1b–d).

The occurence and function of starch phosphorylase isoenzymes have been thoroughly investigated in developing cotyledons of broad beans and leaves of maize [3–5]. In broad bean cotyledons the presence of two phosphorylase isoenzymes was demonstrated. The electrophoretically more slowly moving phosphorylase may be associated with plastids, especially with amyloplasts. The association dissociation equilibrium depends on the salt and sugar concentration, respectively. Sucrose favors the dissociation of this enzyme from the plastids. The extraction buffer used in our experiments usually contained 0.5 M sucrose thus presumably maintaining dissociation conditions. Consequently one may expect this enzyme to

* Part 5 in the series 'The function of the pod for protein storage in the seeds of *Vicia faba* L.'. For part 4 see Müntz, K., Schalldach, I. and Manteuffel, R. (1976) *Biochem. Physiol. Pflanzen* **170**, 465.

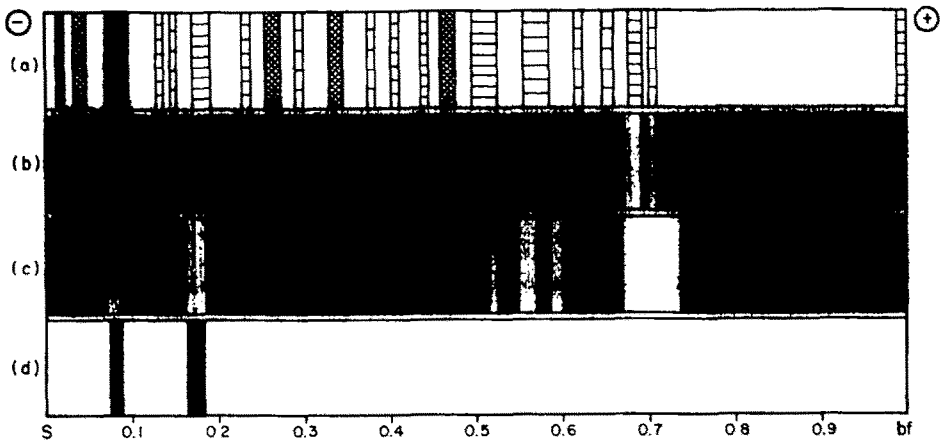

Fig. 1. Patterns of soluble protein and starch degrading enzymes from field bean pods after seperation on 7% polyacrylamide gels, pH 8.9 (a) protein banding pattern after seperation of 75 μg soluble protein; (b) α-amylase double zone after seperation of 50 μg soluble protein and 45 min incubation with Pi free buffer at 37°; (c) starch degradation zones after seperation of 200 μg soluble protein and 3 hr incubation with Pi buffer; (d) phosphorylase mediated starch synthesis after seperation of 100 μg soluble protein and 2 hr incubation with glucose-1-phosphate in otherwise Pi free medium at 37°.

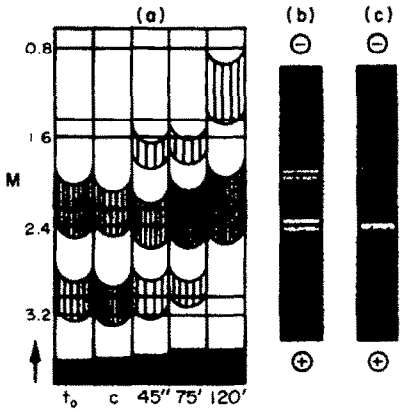

Fig. 2. Identification of α-amylase specificity by starch elution chromatography. (a) Strips from left to right: Pure amylose substrate at time 0 of enzyme action; amylose substrate without enzyme (control); amylose degradation products after 45, 75, and 120 min of enzyme action. The enzyme preparation was eluted from a preparative gel column after electrophoretic separation of soluble proteins. (b) Electrophoretic control of amylase activities of the original extract, and (c) of the eluate from the preparative column.

be dissolved almost quantitatively. In accordance with the findings of De Fekete [8] the more rapidly moving type of phosphorylase not associating with plastids represented the stronger zones on the gels also in our experiments, both in starch degradation and synthesis assays with pod extracts.

Gerbrandy and Verleur [6] also have analysed the patterns of phosphorylase isoenzymes of different organs of *Vicia faba*. Two isoenzymes also occured in developing seeds whereas leaves, roots, and cotyledons of seedlings exhibited some more weak bands than pods in our work and developing cotyledons in the work of De Fekete, respectively.

Interference of the main amylase zones at R_p 0.72 and 0.74 with acid phosphatases as with pea cotyledons [7, 8] could be excluded [9], because no acid phosphatase could be demonstrated at the positions of these amylase bands. Densitometric scanning indicated that the protein zones corresponding to the amylase double zone exhibit the same quantitive changes as the starch degradation bands.

After preparative seperation of soluble pod proteins the main amylase double band was localized, excised from the preparative column, and homogenized in extraction buffer. Subsequently, the homogenate was eluted for 15 hr at 4°. The eluate was used for determination of amylase specificity. Determination of the specificity of the amylase double zone led to the conclusion that it was α-amylase. After 45, 75, and 120 min incubation of pure amylose or potato starch, respectively, with the enzyme eluate from the preparative gel no maltose or glucose could be demonstrated by TLC. Correspondingly, starch eluation chromatography indicated a progressive decrease of high MW glucans and a corresponding increase in groups of iodine stainable dextrins (Fig. 2a).

In inactivation experiments [10] subsequent to the protein separation the gels were heated to 70° for 10 min before incubation with the respective buffer at 37°. After heating amylase activity was preserved at the position of the main amylase zones (R_p 0.72–0.74), thus confirming the chromatographic results.

Amylases in different tissues of the pod

Light microscopical observation of cross sections of the pods from field beans indicated the existence of at least 5 tissue layers, the middle of them representing the bulky mesophyll, and the inner corresponding to aerenchymatic tissue, both tissues containing starch granules. Mesophyll cell layers and the inner aerenchymatic tissues are seperated by sclerenchyma, which made it possible to scrape off the inner cell layers ('endocarp'), and remove the sclerenchyma, and finally to extract inner and outer tissues separately. 60–75% of the outer tissues ('mesophyll') consist of the starch accumulating mesophyll cells.

Both groups of tissues are provided with the same α-amylase isoenzymes. Separating equal amounts of soluble proteins it became evident that the endocarp cell layers contain much fewer amylases than the outer.

The relative activities or quantities of the two isoenzymes, respectively, also appeared to be different. The staining intensity of the corresponding protein bands always exhibited the same pattern as the respective zones of amylase activity (compare Figs. 1a and 1b).

The quantitive differences in amylase activity of the 'mesophyll' and 'endocarp' tissues were paralleled by corresponding differences in the 'starch' content. Mesophyll tissue contained 5 times more insoluble carbohydrates on a dry wt basis than endocarp cell layers from the pod 20 days after anthesis (mean seed length 7 mm). During week 4 and 5 this factor decreased to *ca* 3 by starch degradation in the mesophyll layers whereas the 'starch' concentration of the 'endocarp' remained almost unchanged at a value of 11–12% of the dry matter.

Changes in amylase and phosphorylase patterns during pod development

α–Amylase exhibited phase dependent changes in the isoenzyme pattern during fruit development. During stage 1 [11], up to 3 weeks after anthesis pods contained only one α–amylase. A second α–amylase appeared at stage 2 (week 4 and 5) when starch metabolism in the pod mesophyll cells turned from synthesis to degradation (Fig. 3). At late stage of senescence, starting from week 6, one of these isoenzymes seems to disappear more rapidly than the other, and therefore once more only one α–amylase zone is visible on the gels. By comparison of gels on which extracts from stage–1–pods (one α–amylase zone), from stage–2–pods (two α–amylase zones), and mixtures of these two extracts have been separated, it became evident that α–amylase zone 2 (R_p 0.74) corresponds to the newly appearing enzyme activity.

The relative amounts of the two enzymes proteins and the corresponding amylolytic activities are changing during the period of the presence of both α–amylase isoenzymes. A semiquantitative evaluation of the densitometric curves of amylase activity has also been achieved. The integral of the peak area was used as a relative measure. Maximal activity of the amylases from the main double zone coincides with week 4 and 5 after anthesis, and is in accordance with the period of maximal starch degradation pointed out in our previous communication [11]. At the same period seed length increased from 7 to 11 mm.

No corresponding changes in isoenzyme pattern of starch phosphorylase could be observed. Only quantitative changes were recorded during pod development. Densitometric peak height decreased in senescing pods.

α–Amylase seems to be the only amylase type that can attack starch grains directly [12]. A system regulating starch degradation based on the noncompetitive inhibition of α–amylase by the product of amylolytic starch breakdown, maltose, was demonstrated in maize leaves [13]. In cereal grains starch is reactivated by α–amylase that is synthesized *de novo* during initial germination processes [14]. On the basis of these results we can assume that α–amylase play a major role in the regulation of starch breakdown. This regulation can be medicated either on the enzyme level or on the level of enzyme biosynthesis, including possibly translational and/or transcriptional control.

In pods of developing field beans a new α-amylase zone appears on gel zymograms at the time when the main period of starch breakdown starts. The new starch degradation zone is accompanied by the appearance of the corresponding protein band. On the basis of our present experiments we cannot decide whether the enzyme is produced by *de novo* synthesis or arises from enzyme activation processes.

Fig. 3. Banding pattern of α-amylase from pods of different stages of development: (a) week 3, (b) week 4 and 5, (c) later than week 5 after anthesis.

EXPERIMENTAL

Fruits of field beans were harvested from plants cultivated outdoors or in green houses (*V. faba* L., var *minor*, cv. 'Dornburger'). Flowers were tagged at the probable time of pollination. Only fruits of the flowering nodes 2, 3, and 4 from below were used. Additionally, the developmental stages were characterized by measuring the seed and cotyledon length [2, 11].

Extraction was carried out essentially as in ref. [2] except that the extraction buffer was 0.1 M Tris–maleate buffer, pH 6.9, containing 0.5 M sucrose, 0.02 M $CaCl_2$, and 0.1% cysteine-HCl.

Protein measurement. Soluble proteins were pptd from an adequate vol. of the particle free extract by means of TCA and measured quantitatively using the Folin-reagent [15]. Soluble pod protein pptd and washed with TCA was used as a standard. The calibration curve was based on Kjeldahl estimations [16] of the protein concn in the respective stock soln of the standard protein.

Gel electrophoresis was performed as published previously [17]. The basic discontinuous system was used [18, 19]. In comparison to other starch preparations the sensitivity of the amylase zymography could be increased by *ca* 3 by including only 0.03% of the partially hydrolysed starch according to ref. [20] into the gels of the amylase assay, and 0.01% in the case of phosphorylase assay, respectively.

Zymography. The procedures were based on those described in ref. [21]. Incubation of starch containing gels with Pi buffer led to the appearance of all starch degradation zones that could be differentiated into amylases and phosphorylases using Pi free buffer, 0.1 M citrate–NaOH, pH 5.1, without and with glucose-1-phosphate, respectively, for 45 min or 3 hr at 37°. The corresponding protein zones were made visible by staining with Coomassie Brillant Blue G 250. Protein and zymogram gels were scanned in a densitometer. Preparative separations were achieved using 6% cylindrical gels without starch that have been purified by a 5 hr prerun with separation gel buffer before use. Amylase zones of the preparative gels were localized by placing the column on a starch containing 6% gel (pH 5.1) slab and incubation at 37° for 90 min. At the amylase site a bright zone appeared on the slab after I_2 staining.

Amylase specificity was determined using the differences in

temperature sensitivity of α- and β-amylase [10] and the starch elution chromatography method [22]. Starch elution chromatography was performed by application of starch (control) and starch degradation products after incubation with amylases eluted from the preparative gels to strips of chromatographic paper. Subsequently, the starch was eluted from the start positions by stepwise chromatographic development using $HClO_4$ solns of discontinuously increasing concn (Fig. 2a). starting with pure H_2O. Low MW degradation products are moving more rapidly at lower concn of $HClO_4$ than at the higher ones. Since β-amylases produce maltose and glucoamylases glucose, respectively, only α-amylases give degradation products that can be detected by starch elution chromatography with subsequent I_2 staining. Degradation products were additionally analysed by TLC [23].

Acknowledgements—The author wishes to express his gratitude to Mrs. R. Gillandt for her skilful technical assistance, and to Dr. M. Richter and Dr. S. Augustat from the Zentralinstitut für Ernährung der Akademie der Wissenschaften der DDR, Bergholz-Rehbrücke, for their reliable advice on starch elution chromatography and the gift of a pure amylose preparation.

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