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POLYMORPHISM IN MAIZE, OATS AND SORGHUM α-AMYLASES

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Key Word Index—*Zea mays*; *Avena sativa*; *Sorghum bicolor*; *Hordeum vulgare*; Gramineae; crossed immunoreactivity, α-amylase.

Abstract—Immunochemical characterization of maize, oats and sorghum α-amylase was achieved by use of two immune sera against barley α-amylase isoenzymes. Two distinct antigens were found in oats and sorghum, three in maize, of low pI and similar M_r s.

INTRODUCTION

α-Amylases (EC 3.2.1.1) of cereal seeds provide a typical example of enzyme polymorphism, raising questions on the origin and functional significance of this heterogeneity. Numerous studies on barley and wheat seeds led to the division of α-amylases into two groups, according to their isoelectric points: the 'low pI' group, also called α-amylase I (pIs between 4.5 and 5) and the 'high pI' group, also called α-amylase II (pIs between 5.8 and 6.8), which is the major component [1]. Each group corresponds to a distinct antigen [2] and the different genomic clones described suggest that each group is the product of a family of genes [3, 4]. Functionally, the two groups differ by their specific activity on starch granules, by their sensitivity to an endogenous inhibitor [1] and by the factors regulating their expression [1, 5].

An attempt was recently made to investigate the distribution of *iso*-α-amylases in germinating seeds of a number of other cereals [6]. The analysis was essentially based on the charges of α-amylase constituents, by means of isoelectric focusing (IEF) and chromatofocusing. The absence of high pI α-amylase in some cereals and the presence of intermediate constituents in others were established. However, the cause of the absence of one α-amylase group (lack of the corresponding genes, or mutations changing the charges of the expressed proteins) as well as the identification of the intermediate constituents remains unknown.

This work was undertaken to analyse further the grouping of the different α-amylase constituents in terms of antigenic relationship in maize, oats and sorghum. Two available immune sera, respectively against α-amylase I and α-amylase II of germinating barley seeds were tentatively used, since some antigenic relationship between the enzymes of the four cereals considered had been reported [6–8].

RESULTS AND DISCUSSION

α-Amylase zymogram patterns were examined after IEF separation on a pH 4–9 gradient (Fig. 1). Compared to barley α-amylases, maize, oats and sorghum α-amylases consisted of only low pI forms (pI < 5.8), as previously described by MacGregor *et al.* [6] for other cultivars. The chromatofocusing experiments of these authors indicated only one major peak of activity for sorghum (pI 4.3), a broad group between 4.4 and 5.1 for oats, and for maize a main peak at 4.9 and minor components at pI 4.3 and at pI 5.5–5.8. The zymograms obtained here in IEF are in good agreement with these values, showing only one group of bands for sorghum and oats and several for maize. A narrower gradient (4–6) was thus used thereafter, in order to expand the α-amylase zone of the three cereals.

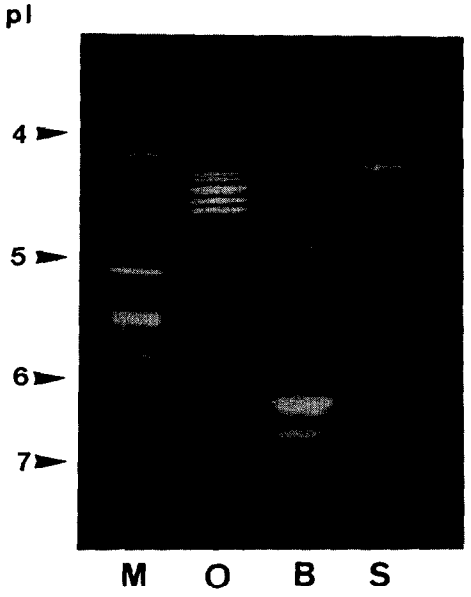

Fig. 1. IEF of cereal extracts (1 Unit/lane), stained for α-amylase activity. M: Maize; O: oats; B: barley; S: sorghum.

Use of barley α-amylase antisera for characterization of maize, oats and sorghum isoenzymes

The immune sera were tested by rocket immunoelectrophoresis performed during a short time, to determine whether antibodies reacted with all forms of the

investigated cereals, and to know whether one or several antigens were characterized. At pH 8.6, with a non-immune serum in the gel, all α-amylases migrated towards the anode, and appeared after staining as white spots on a dark background (Fig. 2a). With an equimixture of barley α-amylase antisera, the corresponding area of the gel was free of α-amylase activity, which was trapped in the rocket immunoprecipitates appearing below (Fig. 2b). This indicated that every form of detected enzyme reacted with barley α-amylase antibodies. These antibodies were thus validated for the study of oats, sorghum and maize α-amylases.

Characterization of immunologically distinct α-amylase forms

After rocket immunoelectrophoresis (Fig. 2b, c), two superimposed peaks were observed for oats and sorghum, indicating the détection of two distinct antigens, as for barley, in these cereals. For maize, three superimposed rockets were visualized, the upper one, very faint, showing a low homology. Three antigens were thus detected. α-Amylase from germinating oats, sorghum and maize, though of low pI only, appeared therefore to be composed of several antigenically distinct forms.

In order to correlate pI and antigen distribution, cross-immunoelectrophoresis was performed with each immune serum separately (Fig. 3). As the gradient and extract amounts differed from the experiment shown in Fig. 1, more bands were visualized after the IEF step. Each anti-barley immune serum contained antibodies reacting with each antigen from maize, oats and sorghum. Based on the comparison of their aspect after staining for α-amylase activity (well formed or diffuse), confirmed by protein staining (not shown), the antigen showing the best reaction with each immune serum was easily identified. This identification is reported on the corresponding figure. For oats and sorghum both antigens corresponding to α-amylase I and α-amylase II were overlapping (Fig. 3a, b). In maize they were identified in separate zones of the IEF diagram (Fig. 3c). The 'α-I type' antigen always corresponded to the lowest pIs, but it must be emphasized that all pIs were very close. The observed disposition of antigens for sorghum (pIs 4, 3) differed from the two well-separated antigens (of higher pI: 4.65 and 5.2) observed by Mundy [8]. A comparative study of other cultivars would be of interest, as sorghum cultivars display very different morphologies. For maize, the additional third antigen was identified as the most basic constituent, for which no immunoprecipitate was seen after an overnight electrophoresis (Fig. 3c). Short-run electrophoresis in the second dimension confirmed that the corresponding bands were reacting, though weakly, with the sera (results not shown).

Size heterogeneity of maize, oats and sorghum isoenzymes

The two antigens of barley are known to have slightly different M_rs, the high-pI one being smaller [9]. Immunoblotting following SDS-PAGE was performed on crude cereal extracts, with both antisera, to detect possible size heterogeneity in maize, oats and sorghum. Two bands of close M_r were clearly observed for maize and oats, with a

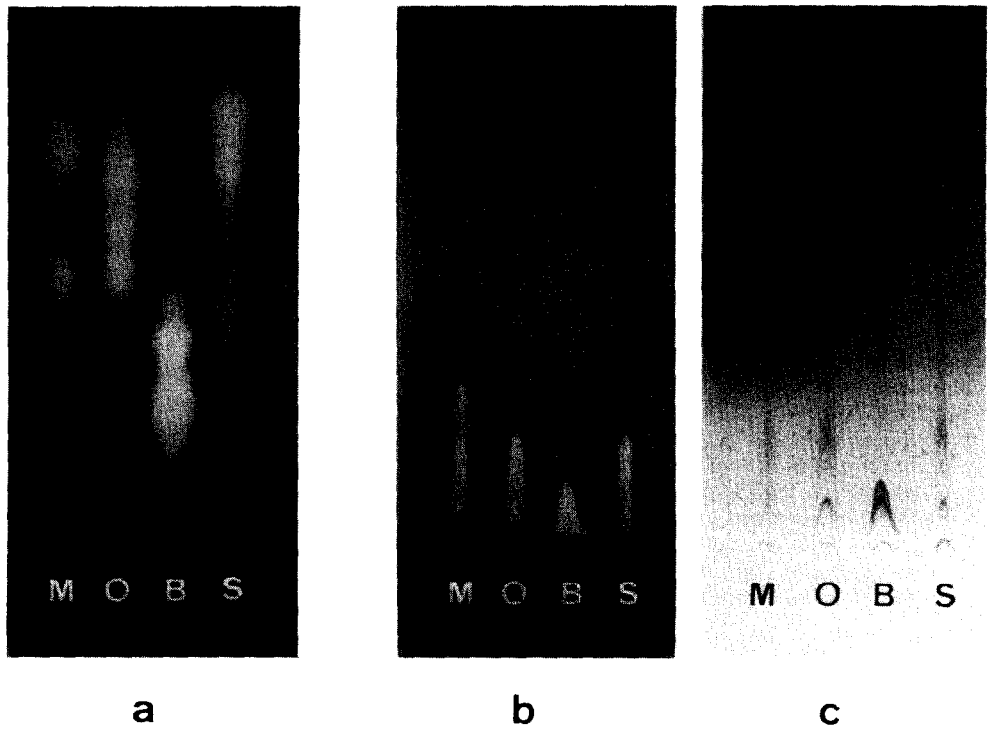

Fig. 2. Rocket immunoelectrophoresis (3.5 hr, 6.5 V/cm) of cereal extracts. The deposited amounts were: for maize (M) 1 Unit, for oats (O) 1.5 Units, for barley (B) 2.5 Units, for sorghum (S) 1.5 units. a: non-immune serum (5% v/v), stained for α-amylase activity. b: equimixture of anti-barley α-amylase I + anti-barley α-amylase II sera (2.5% v/v each), stained for α-amylase activity. c: Same gel as in b, stained for proteins.

gap of *ca* 1500 for maize and *ca* 1000 for oats (Fig. 4). Extensive cross-reactions between each denatured isoenzyme and the sera brought some complexity, but it could be stated that the 'α-II type' (thus with higher pIs) corresponded to the smaller antigen as in barley, as only one band was observed with the corresponding serum. For sorghum only one band was clearly seen with both sera, indicating similar M_rs for both antigens.

CONCLUSIONS

Cross-reactions between cereal α-amylases and two barley α-amylase antisera, apart from indicating common antigenic determinants, allowed the study of some characteristics of maize, oats and sorghum α-amylases without enzyme purification. Although of close low pI, they are composed of several distinct antigens (two for

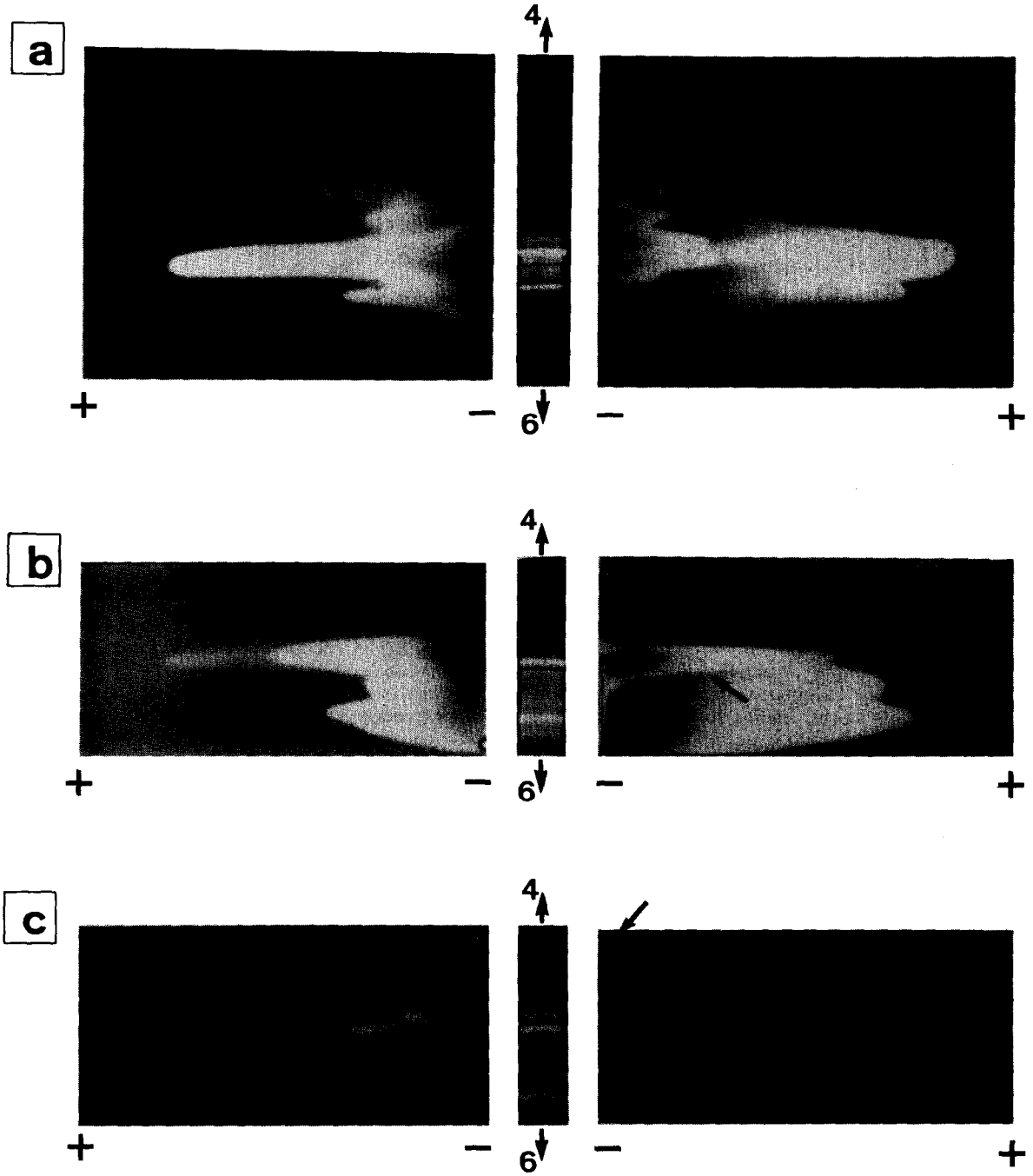

Fig. 3. Cross-immunoelectrophoreses of oats (a), sorghum (b) and maize (c) extracts, stained for α-amylase activity. Right: 2nd dimension with barley α-amylase I antiserum; Left: 2nd dimension with barley α-amylase II antiserum. Four units were deposited for the IEF of each cereal. The amount of sera were 0.5% (v/v) for oats, 2% (v/v) for sorghum and 4% (v/v) for maize. The second dimension electrophoresis was run overnight at 5 V/cm.

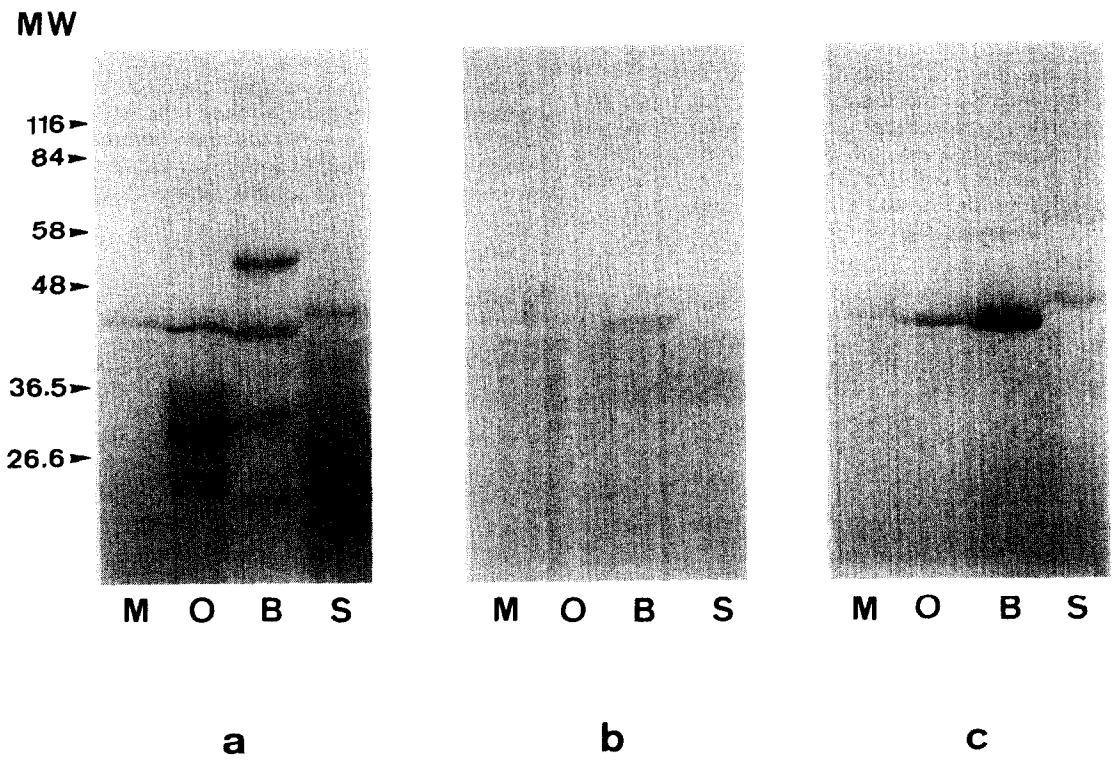

Fig. 4. SDS-PAGE and immunoblotting of cereal extracts; M, O, B, S as in Fig. 1; MW: molecular weight markers. a: Coomassie staining. b: immunoblotting with anti-barley α-amylase I. c: immunoblotting with anti-barley α-amylase II. For a, 12 Units/lane were deposited. For b and c, 15 Units were deposited for maize and sorghum, 10 Units for oats, and 7.5 Units for barley.

oats and sorghum, three for maize), of slightly different M_rs.

The 'high pI' group is not antigenically absent in these cereals, but displays an apparent shift of isoelectric points towards the low values, leading to nomenclature difficulties.

EXPERIMENTAL

Plant material and extraction. Seeds of barley (*Hordeum vulgare* L., cv Menuet), oats (*Avena sativa* L., cv Avalanche), maize (*Zea mays* L., cv Dea) and sorghum (*Sorghum bicolor* L., cv Oasis) were supplied by the INRA-GEVES (La Minière, Guyancourt, France). The grains were surface-sterilized for 30 min with 1% NaClO and allowed to germinate on H_2O saturated cottonwool in the dark, at 20° for barley and oats and at 30° for other cereals. After 6 days the germinated kernels were harvested, roots and shootlets were cut off, and 1 g of the grains homogenized in a mortar with 5 ml 100 mM NaCl, containing 1 mM $CaCl_2$. After 30 min at room temp., the extracts were centrifuged at 10000 *g* for 10 min and filtered through 0.45-μm pore membranes (Millex-HV, Millipore). When required, the extracts were 2- to 4-fold vacuum-concd, to allow enough enzyme activity in immunoelectrophoreses.

Immune sera. The two immune sera were the barley α-amylase I antiserum and the barley α-amylase II antiserum previously used in ref. [10]. Each was obtained by immunisation of a rabbit with the corresponding purified isoenzyme. Though α-amylase I and II are antigenically distinct, some cross-reaction is present between α-amylase I isoenzyme and α-amylase II antiserum and conversely [2]. A control was performed with serum from non-immunised rabbits.

Analytical methods. α-Amylase activity in cereal extracts was assayed by means of the Phadebas amylase test, as modified for cereal α-amylases in ref. [11]. The specific staining of gels for α-amylase activity was achieved with β-limit dextrins as a substrate and I_2–KI reaction. IEF of crude cereal extracts was carried out in 2 mm thick polyacrylamide gels, containing 4% carrier ampholytes: Servalyt 4–9 T (Serva) or Ampholines 4–6 (Pharmacia-LKB). After electrophoresis, gel samples were subjected to either specific zymogram staining or cross-immunoelectrophoresis as previously described [12].

Rocket immunoelectrophoresis [13] was performed on crude extracts and the plates stained for α-amylase activity, then for proteins with Violet 49 (Serva).

SDS-PAGE was performed as described in ref. [14] in 12% acrylamide gels, and followed either by Coomassie staining or by immunoblotting according to ref. [15].

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