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Improved detection of amylase activity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with copolymerized starch

An improved method, based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for detection of amylase activity is described. This method will allow better characterization of certain amylases than that obtained by the Davis technique. The main features of the technique are: (i) identification of amylase bands and molecular mass determination are possible in the same gel; (ii) the hydrolysis of copolymerized substrate during electrophoretic separation is prevented using very low temperatures instead of inactivating agents such as chelating agents; and (iii) the technique is applicable to reveal amylase activity in a wide range of biological samples. The method is not useful for enzymes sensitive to SDS and for high molecular mass amylases.

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PAGE according to Davis [4] has been used extensively in the characterization of amylases. Modifications of the technique are mainly based on the way the substrate is made available to the enzyme: by submersion of the carrying polyacrylamide (PA) gel in a starch solution [1, 2] or by direct contact with a starch-agarose [13] or starch-PA gel [4]. Substrate-PAGE followed by incubation in a starch solution has also been utilized [15]. In all cases, activity is revealed using iodine solutions at variable concentrations. The main limitation of these methods is the difficulty in determining relative molecular masses. The method proposed in the present paper allows the detection of amylase activity from a variety of samples using copolymerized starch gels to perform electrophoresis under SDS conditions according to Laemmli [8]. The main advantage of the method is the possibility of revealing simultaneously the amylolytic activity and relative molecular masses of proteins. Protein bands can be visualized since the presence of starch in the gel does not interfere with Coomassie Brilliant Blue staining.

N,N'-methylenebisacrylamide, Coomassie Brilliant Blue (R-250), SDS, α-amylase from porcine pancrease, α-amylase from *Bacillus* sp. and standard molecular mass markers for electrophoresis (MMM) were supplied by Sigma (St. Louis, MO, USA); tris(hydroxymethyl)aminomethane (Tris), *N,N,N*',*N*'-tetramethylethylenediamine (TEMED) and ammonium persulfate were supplied by Pharmacia Biotech (Uppsala, Sweden). Acrylamide was from Bio-Rad (Richmond, CA, USA). All other reagents were purchased in the purest form available. The power supply used was a Pharmacia EPS 3500 (Pharmacia). Electrophoresis of the enzyme preparations was performed in 8 × 10 × 0.75 cm gels in a Miniprotean II (Bio-Rad) electrophoresis chamber. Amylase extracts were prepared as follows. Rumen amylases: ruminal fluid was sampled from nonlactating female adult sheep using an ororuminal probe. After centrifugation (2000 × *g*, 15 min, 4°C) the pellet containing particle-associated microorganisms was resuspended in distilled water and distrupted by sonication (Microson XL200; Misonix, Farmingdale, NY, USA) for 2 min in 15 s periods. Active extracts were obtained after renewed centrifugation (20 000 × *g*, 15 min, 4°C). Amylases from *Rhynchophorus ferrugineus* (Olivier, 1790) (*Coleoptera*:*Curculionidae*): after dissection of 20-day-old larvae, digestive tracts were homogenized in distilled water (100 mg/mL), disrupted by sonication (15 s) and centrifuged (20 000 × *g*, 15 min, 4°C). Supernatants were used for the assays. Commercial amylases (1 mg/mL) were prepared in distilled water, and diluted to appropriate concentrations for the assays. Amylase activity was measured according to the procedure described by Robyt and Whelan [11]. Different amounts of enzyme extract were mixed with 125 μL of substrate (2% w/v soluble starch) and 125 μL of 0.1 M phosphate-citrate-0.05 M NaCl buffer, pH 6. The mixture was incubated for 15 min at 39°C, and the liberated reducing sugars were measured. One amylase activity unit was measured as the

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Abbreviations: MMM, standard molecular mass markers for electrophoresis; **PA**, polyacrylamide

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quantity of enzyme that releases from soluble starch a quantity of reducing sugars that produce, per minute, the same increase in absorbance (measured at 600 nm) as one μg of maltose under specific assay conditions.

Electrophoresis under native conditions was carried out according to Davis [4]. Samples were diluted 1:1 in sample buffer (0.125 M Tris; 20% v/v glycerol; 0.04% v/v bromophenol blue, without β-mercaptoethanol) and were not boiled. For substrate-SDS-PAGE, 5 μL of MMM were loaded on each plate, and samples were diluted 1:1 in sample buffer (0.125 M Tris, 20% v/v glycerol, 0.04% v/v bromophenol blue, 2% w/v SDS, without β-mercaptoethanol) and were not boiled. The stacking and resolving gels were prepared according to Laemmli [8], using the quantities indicated in Table 1. PAGE with copolymerized starch was prepared in the same way, except that SDS is replaced by distilled water, and sample buffer contained no SDS. Ten μL of the mixture were added to each well (about 0.75 activity units) in all cases.

The technique is based upon the observations that enzymes may be resolved in SDS-PAGE according to their molecular mass, and that amylolytic activity can be revealed in a starch-PA gel because: (i) starch is a sensitive and satisfactory substrate for amylase activity detection, and (ii) when starch is copolymerized at the suggested concentration (0.2% w/v) into the matrix of the SDS-PAGE at the time of casting, it is retained during subsequent electrophoresis of enzyme samples, thus providing *in situ* substrates for previously separated bands of amylase activity.

Substrate-PAGE and substrate-SDS-PAGE were carried out in two phases. Gels were first subjected to a constant voltage of 30 V per gel until the tracking dye (bromophenol blue) entered the separating gel (approximately 30 min). After this, gels were subjected for 75 min to a constant voltage of 100 V per gel. Electrophoresis was carried out at constant low temperature (0–2°C) to avoid starch hydrolysis and the loss of resolution. After electrophoresis, gels were washed in distilled water and then incubated in 0.1 M phosphate-citrate and 0.05 M NaCl buffer, pH 6, for 2–3 h at 39°C. Finally gels were washed and fixed in 12% TCA for 10 min, prior to staining with Lugol solution (6.7 mg/mL KI and 3.3 mg/mL I_2; Fig. 1).

Table 1. Substrate-gel preparation in the SDS-PAGE discontinuous buffer system

Stock solution	Stacking gel (4%)	Resolving gel (12%)
Polyacrylamide[a)]	0.65 mL	4.0 mL
0.5 M Tris-HCl buffer, pH 6.8	1.25 mL	–
1.5 M Tris-HCl buffer, pH 8.8	–	2.5 mL
Distilled water	3.05 mL	2.25 mL
2% w/v soluble starch[b)]	–	1.25 mL
10% w/v SDS	50 μL	100 μL
TEMED	5 μL	5 μL
10% w/v Ammonium persulfate[c)]	25 μL	50 μL

a) Acrylamide: Bis 30:0.8 w/w in distilled water, filtered through Whatman No. 1 filter paper and stored at 4°C
b) Solubilized in water using a microwave oven
c) Prepared immediately before use

Initially, substrate-SDS gels were washed and gently shaken in 2.5% w/v Triton X-100 for 1 h at 4°C to remove the SDS and restore enzyme activity after electrophoresis was carried out, according to Lacks and Springhorn [7]. This step was later deleted without observing any negative effect on zymogram resolution produced by persistence of SDS in gels. Samples were evaluated in PAGE according to Davis [4] (Fig. 2). After electrophoresis gels were imbibed in 0.5% w/v starch at 4°C for 45 min to assure starch penetration into the acrylamide matrix. Gels were then incubated for 2–3 h at 39°, after which they were stained with the previously described Lugol solution. After staining, amylase bands were detected as clear yellow zones against a dark brown background of nonhydro-

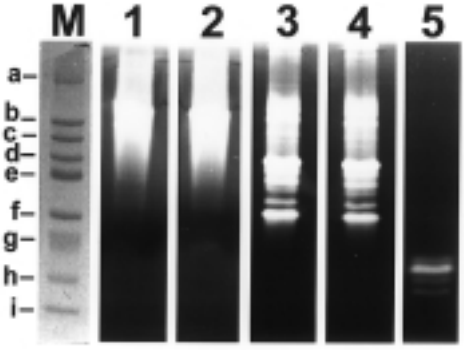

Figure 1. Starch-PAGE and starch-SDS-PAGE zymograms of extracts prepared according to the methods described in the text. Lane (M) MMM: a, phosphorylase *b* from rabbit muscle (97 kDa); b, fructose-6-phosphate kinase from rabbit muscle (84 kDA); c, bovine serum albumin (66 kDa); d, glutamic dehydrogenase from bovine liver (55 kDa); e, ovalbumin from chicken egg (45 kDa); f, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa); g, carbonic anhydrase from bovine erythrocytes (29 kDa); h, trypsinogen from bovine pancreas (24 kDa); i, trypsin inhibitor from soybean (20 kDa); j, α-lactalbumin from bovine milk (14.2 kDa); k, aprotinin from bovine lung (6.5 kDa); (1), (2) enzymatic extracts of ruminal microorganisms in starch-PA in native gels according to Davis [4]; (3), (4) enzymatic extracts of ruminal microorganisms in starch-SDS-PAGE according to Laemmlil [8]; (5) *Rhynchophorus ferrugineus* larval extracts in starch-SDS-PAGE.

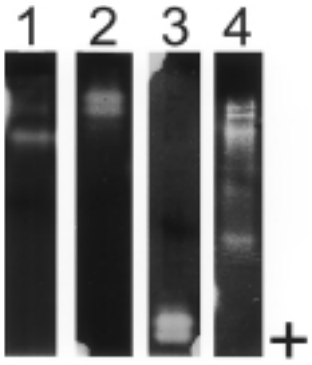

Figure 2. PAGE zymograms of extracts prepared according to Davis [4] and incubated in soluble starch as described in the text. Lane (1) α-amylase from *Bacillus* sp.; (2) α-amylase from porcine pancrease; (3) amylases from *Rhynchophorus ferrugineus* larval extracts; (4) amylases from enzymatic extracts of ruminal microorganisms.

lyzed stained starch. Iodine staining is not permanent in gels and must be photographed a few minutes after iodine treatment (a Ricoh KR-10 camera, box light, macro objective and Kodak 5052 T-Max 100 black and white film were used). However, it is possible to maintain gels indefinitely when they are permanently immersed in an iodine solution hermetically stored at 4°C. Iodine solution avoids deterioration of stored gels due to spoilage by microbiota.

SDS-PAGE allows the determination of relative molecular masses by densitometric analysis (using TDI-1D Version 2.0 software; TDI S.A., Madrid, Spain), and comparisons with a standard molecular mass. The proposed procedure does not allow visualization of MMM after iodine staining, but this can be overcome in two ways. (i) A slice containing the lane with the MMM can be cut and stained with 0.1% w/v Coomassie Brilliant Blue R-250 in a methanol-acetic acid-water solution (40:10:50) and left overnight at room temperature; destaining with methanol - acetic acid - water (35:10:55). (ii) Once it has been iodine-stained and photographed, the whole gel can be submerged in Coomassie stain, following the procedure described above. Neither starch nor iodine interfere with protein staining and thus protein bands can also be characterized. Special attention must be paid during electrophoresis when substrate is copolymerized in the gel matrix. When proteins are separated in gels containing high molecular mass substrates, such as starch, migration can be affected by the resistance offered by such substrates [5], as can be observed in substrate-PAGE for ruminal amylases (compare Fig. 1, lanes 1 and 2 with Fig. 2, lane 4). It is highly recommended that both enyzmatic samples and MMM are carried out in the same gel, avoiding possible differences in migration produced when MMM are carried out in parallel electrophoresis, due to minimal differences in substrate or PA concentrations between gels.

Assayed enzymatic extracts were tested in relation to their possible SDS inactivation. A one % SDS concentration (ten times used in electrophoresis), maintained for 60–120 min, produced only a 20% reduction in amylase activity for enzymatic extracts from ruminal microorganisms (Fig. 3). SDS resistance seems to be a general feature of polysaccharides of anaerobic origin [1], and such property could result in a technological advantage [2, 9, 10]. In fact, SDS was not able to inhibit the amylase activity of the extracts (Fig. 1, lanes 3 and 4) during enzyme migration in starch-PA gels when the electrophoresis chamber was maintained at room temperature. A similar lack of effect was observed for *R. ferrugineus* extracts. Enzymes used as controls (*i.e.*, porcine and *Bacillus* sp. α-amylases) were inactivated in the presence of SDS, and dramatic artifacts were observed during SDS-electrophoretic migration. Commercial SDS-resistant control enyzmes were not available.

Although not studied here, possible methods of preventing enzymes from acting on their substrate are: (i) separation is carried out in the presence of denaturing agents (SDS), and after separation the gel is washed in buffers that are free of detergent or contain cationic detergent, allowing protein renaturalization [5], and (ii) use of chelating agents to bind an essential metal ion for enzyme activity (*e.g.*, calcium), and after completion of electrophoresis the gel is incubated in a buffer containing such metal ion in a concentration that overcomes the effect of the chelating agent [10]. However, on complete removal of Ca(II), some enzymes are fully active but are much less stable, and their role in α-amylase is to maintain the stability of

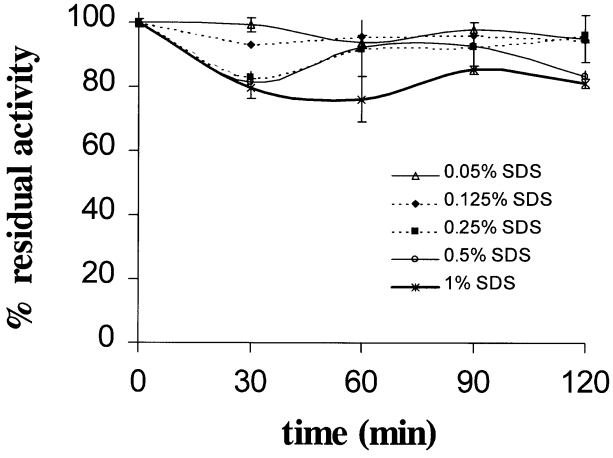

Figure 3. Effect of SDS concentrations (w/v) on amylolytic activity of ruminal enzymatic extracts at different preincubation times.

the secondary and tertiary structure. There is no evidence to indicate that Ca(II) plays a direct role in binding or transforming the substrate [14], particularly in some anaerobic microorganisms [2].

To prevent enzymes from acting on their substrate, the running temperature has been decreased in the proposed method. A very low temperature was maintained (0–2°C) inside the electrophoresis chamber in order to avoid hydrolysis of starch in gel lanes while electrophoresis was carried out and enzymes were migrating within the gel. If not, staining with iodine resulted in "trails" in which it was not possible to define amylolytic bands. Even when the electrophoresis unit was maintained in the refrigerator at 4°C, trails of starch degradation appeared; electrophoresis must therefore be performed in a freezer or under other cooling conditions able to provide temperatures below 0°C inside the electrophoresis chamber. Heat generated by the electric flux in the electrophoresis chamber and saline concentration of electrophoresis buffer prevent gels from freezing during the electrophoresis. The low temperature allows the enzymes to migrate inactively, without starch hydrolysis taking place simultaneously. The final incubation at 39°C allows the amylase enzymatic activity to be expressed. The apparition of such "trails" could be overcome by further incubation of gels in buffer enriched with soluble starch [15]. However, the addition of extra quantities of substrate during the incubation period could result in losses of amylolytic bands of low enzymatic activity, due to the incorporation of new starch into the PA matrix. That could hide the visualization of amylase bands weak in enzymatic activity. On the contrary, the incubation of gels with a fixed starch concentration in buffer with no substrate makes the method more sensitive.

Limitations of the technique: When the procedure described was used for the electrophoretic analysis of different sources of amylase enzymes (Fig. 1, lanes 3–5), the method resolved clearer bands of enzymatic activity than the Davis method [4] (Fig. 1, lanes 1 and 2). However, the procedure does have some limitations. When gels were prepared at PA concentrations lower than 10%, there were some difficulties in retaining starch in the PA matrix, and starch tended to escape from gels, producing a loss in background uniformity of gels when they were stained. This represents a limitation to resolve high molecular mass amylolytic enzymes that should be performed in gels containing less than 10% PA. In addition, as previously described, copolymerized high molecular mass substrates in slab gels can affect separation of protein [5], limiting especially the resolution of high molecular mass amylases. This phenomenon could be partially overcome by using a β-mercaptoethanol buffer [2, 5] for some amylases, allowing enzyme subunits to be separated and visualized. Obviously, the inactivation of amylolytic activity by SDS is the other main limitation of this method in characterizing amylases.

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