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Simplified Electrophoretic Assay for Human Salivary α-Amylase Inhibitor Detection in Cereal Seed Flours

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α-Amylase inhibitors are antinutritional proteins largely found in cereal seeds. An in-gel assay was developed that allowed the rapid screening of these compounds in complex seed extracts. The assay was based on the electrophoretic separation of the extract proteins on starch-containing gels, followed by the detection of α-amylase-inhibiting proteins after incubation of the gel in an α-amylase solution; inhibitors were revealed by a staining method based on iodine binding to nondigested starch. The one-dimensional method can be useful to test inhibitory activity of purified proteins or to assay fractions recovered during a purification procedure. A two-dimensional (IEF × PAGE) non-denaturing system with second-dimension separation on starch-PAGE was also developed; the technique allowed the screening of complex protein mixtures for multiple inhibitory proteins. The newly developed assay method was used to test the presence of inhibitory activity in a crude extract from wheat flour, and it was validated by comparing in-gel and in-solution assays of commercially available α-amylase inhibitors.

KEYWORDS: α-Amylase inhibitor; in-gel assay; starch-PAGE; non-denaturing 2-DE

INTRODUCTION

α-Amylase inhibitory activities have been detected in salt extracts of flours obtained from several *Triticum* species (*1*, *2*). These proteinaceous inhibitors are coded by multiple genes and are members of the large plant trypsin/α-amylase inhibitor family, which includes monomeric, homodimeric, and heterotetrameric α-amylase inhibitors. By virtue of differences in their primary structures, these proteins are able to inhibit to different extents a wide range of α-amylases from several sources (*2*−*6*). This target heterogeneity is possibly linked to the protective role that these inhibitors play against various pests, allowing for a wide defense spectrum. Among the target enzymes are mammal α-amylases, included those from human saliva and pancreas.

Plant inhibitors of α-amylases have been the subject of many studies both for the regulatory role they play on the endogenous enzymes and for their nutritional and toxicological properties; in fact, besides a possible deleterious effect on sugar assimilation, these proteins are the well-known antigens responsible for the allergic disease baker's asthma, provoked by the inhalation of cereal flour (*7*, *8*). The interest in these proteins is also based on their exploitation as insect pests repellent and the possibility to engineer transgenic crops expressing the protective proteins (see ref *9* for a review on the topic).

The current methods of α-amylase inhibitor assay generally employ an in-solution approach based on the measurement of the decrease of amylase activity on a starch substrate (*10*−*12*); however, these quantitative assay methodologies are either time-consuming, especially when used to monitor the progresses of a purification process (e.g., if chromatography fractions are to be tested), or not informative enough, that is, in the case of the analysis of complex protein mixtures. An electrophoretic method for the detection of the α-amylase inhibitors from cereal and legume seeds has been described by Giri and Kachole (*13*); however, on their gels, protein band resolution seemed to need improvement, and a 2-DE method, able to resolve the multiple forms of the amylase inhibitors, had not been described. Moreover, the authors were not able, at that time, to recover the inhibitory activities on SDS-containing starch-PAGE gels.

In the present work, we describe an in-gel inhibition assay that allows for the analysis of multiple samples at one time, permitting the visualization, either on non-denaturing or denaturing PAGE, of the α-amylase inhibitory components of protein mixtures. Moreover, by applying the method described to two-dimensional electrophoresis (2-DE; IEF × PAGE), it is possible to resolve single inhibitory components in complex mixtures without the need to purify them and to isolate and identify the inhibiting spots. Hence, the assay can be a useful tool for the analysis of the α-amylase inhibitor proteome of flours and the discovery of potential allergens and protective proteins. The results presented are also discussed in comparison with those obtained with the analogous method described by Giri and Kachole (*13*).

MATERIALS AND METHODS

Plant Material. Seeds of the soft wheat *Triticum aestivum* cv. Centauro were purchased from the Società Produttori Sementi S.p.A.

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(Bologna, Italy). The seeds were ground in a break roller-mill (Labormill 4RB, Bona, Monza, Italy), and the flour was stored at −20 °C until used.

Inhibitor Sources. The source for the investigation of α-amylase inhibitory activities was a salt-soluble protein extract from soft wheat (*T. aestivum* cv. Centauro) flour. Salt-soluble proteins were extracted by continuosly stirring 10 g of wheat flour at 4 °C with 50 mL of 0.15 M NaCl, for 1 h, and centrifuging the extract at 16400*g* for 15 min at 4 °C. The extraction was repeated twice, and the supernatant proteins were precipitated for 30 min at 4 °C with 50% saturation ammonium sulfate. After centrifugation as already described, the precipitate was resuspended with 10 mM ammonium acetate buffer, pH 7.5, and dialyzed overnight against 20 volumes of the same buffer. The precipitated samples to be used for two-dimensional separations were resuspended and extensively dialyzed against 1000 volumes of water.

The sample was treated at 100 °C for 5 min to inactivate possible endogenous amylolytic activities; after centrifugation to remove the precipitate, it was divided in aliquots and stored at −20 °C.

Commercial sources of α-amylase inhibitory activity were inhibitors type I and type III from *T. aestivum* seed (Sigma-Aldrich, Inc., St. Louis, MO).

Protein Determination. The flour extract protein content was measured with the Bradford method (*14*).

In-Solution α-Amylase Assay. The α-amylase activity was estimated with the Bernfeld method (*10*) by measuring the release of maltose from soluble potato starch (Carlo Erba, Milan, Italy) in 20 mM sodium phosphate buffer, pH 6.9, containing 6.7 mM sodium chloride (assay buffer). One unit of α-amylase was defined as the amount of enzyme that released 1.0 mg of maltose from starch in 1 min at pH 6.9, at 37 °C. A maltose calibration curve was generated with potato starch under the same conditions used for the α-amylase assay and used as reference.

The assay was used to test the activity of α-amylase (EC 3.2.1.1) from human saliva, from *Bacillus subtilis* (type II-A), and from barley (*Hordeum vulgare*) malt (type VIII-A) (all from Sigma-Aldrich). All of the enzyme activities were tested for linearity and substrate saturation; assay working conditions were chosen so that the enzymes were saturated with starch. All of the assays were performed in triplicate.

In-Solution Inhibition Assay. When the wheat salt-soluble extract or the commercial inhibitors were tested for α-amylase inhibitory activity, all α-amylases were added at 0.05 unit/assay volume. The protein sample to be tested was preincubated with the enzyme for 30 min at 37 °C before the assay was begun by adding the starch substrate, as described above. α-Amylase activity control assays were run by preincubating the enzyme with an equal volume of assay buffer to compensate for the "inhibitor" volume. All of the assays were performed in triplicate.

1-DE and 2-DE Assays of α-Amylase Inhibitors. All gel slabs used were PAGE minigels (*15*) incorporating 0.1% soluble starch as α-amylase substrate; they were 0.5 mm thick for 1-DE or 0.75 mm thick for 2-DE. Starch-incorporating gels (starch-PAGE) contained 7.5% acrylamide, whereas SDS-starch-PAGE contained 12% acrylamide and 2% SDS; they were all cast based on our modification of the method described by Martínez et al. (*16*).

The samples to be tested for inhibitory activity on 1-DE were mixed with non-denaturing electrophoresis sample buffer (5×) and subjected to electrophoresis at room temperature with low current (5−7 mA/gel) and constant voltage (300 V), for about 2 h. At the end of the run, the gels were sliced, and each sample-containing gel strip was incubated with 6 units of human salivary α-amylase in 2 mL of assay buffer (see above) at 37 °C for 1 h. The gel strips were rinsed with distilled water and fixed for 10 min in 10% TCA. After another rinse with water, the strips were stained with Lugol's reagent (6.7 mg/mL KI and 3.3 mg/mL I_2). α-Amylase inhibitors were revealed as dark bands where the starch was not digested by the enzyme. Immediately after the electrophoresis run, a duplicate of each gel strip was placed in CBB R-350 (PhastGel Blue R; Amersham Pharmacia Biotech AB, Uppsala, Sweden) to be stained for protein.

In the case of SDS-starch-PAGE, the gels were placed in a 2.5% (v/v) Triton X-100 solution for 30 min and rinsed with water, before being incubated with α-amylase.

The first dimension separation of the starch-PAGE 2-D gels was carried out on 7 cm IPG strips (Bio-Rad Laboratories, Inc., Milano, Italy), pH range 5−8. The IPG strips were rehydrated overnight with 100 μg of protein sample resuspended in 125 μL of reswelling buffer (10% sucrose, 0.2 M taurine, 2% IPG buffer, pH 3−10, and traces of BBP). The first-dimension separation was carried out with a Multiphor II unit (Amersham Biosciences AB) using the following electric parameters: 300 V for 30 min, followed by a 1 h linear increase to 1000 V and by a 2 h step at 2000 V. The strips were equilibrated in non-denaturing sample buffer for 20 min under constant mild shaking, rinsed, and applied on a 7.5% starch-PAGE minigel; 5% agarose in electrophoresis buffer was poured over the strip. The second-dimension electrophoretic run was performed as described above. The gel was incubated for 1 h at 37 °C in 20 mL of assay buffer containing 90 units of human salivary α-amylase, fixed in TCA, and stained with Lugol's reagent. For each gel stained for amylase activity, a duplicate gel was stained for proteins with CBB R-350. All 2-D gels were made in triplicate.

RESULTS AND DISCUSSION

In the present work we describe a method for the detection of human salivary α-amylase inhibitors after separation on 1-DE and 2-DE on starch-containing gels.

In-Solution α-Amylase Inhibition Assay. Preliminary in-solution assays were carried out to verify the presence of amylase inhibitory activities in the flour extract and to compare them against wheat commercial inhibitors. The optimal amount of amylase activity to be used in the assays was determined with saturation assays for each of the three amylases tested (**Figure 1A**), and the common value of 0.05 unit/assay volume was chosen. When the effect of several concentrations of boiled flour extract and boiled commercial inhibitors was tested on the three different amylases (**Figure 1B**), an increasing inhibition of the human salivary amylase was observed for all of the inhibitors tested; on the other hand, neither the amylase from *B. subtilis* nor the enzyme from *H. vulgare* was significantly affected by the wheat commercial or noncommercial inhibitors. Inhibitor type I is a mixture of the three molecular mass families (60, 24, and 12 kDa) of cereal trypsin/α-amylase inhibitors, whereas inhibitor type III contains inhibitors belonging to the 24 kDa family only. It was estimated that 5 μg of protein should have been sufficient to show amylase inhibition by the commercial inhibitors, due to the purity of these preparations. Besides the difference in inhibitor purity, the lower concentration tested with respect to that used for the wheat extract was also based on the knowledge that these inhibitors are not usually effective against bacteria and endogenous α-amylases (*17*).

Cereal seeds and the flours obtained by their processing are rich in starch and may contain endogenous amylase activities. To ensure that none of these compounds was present and active in the flour extract used in our study, the sample was kept in a boiling water bath to inactivate unwanted hydrolytic enzymes that could compete with the assay amylase. Even though this process may have determined the possible loss of inhibitory activities, after heating, we were able to recover a strong amylase inhibition. As a control, the commercial inhibitors were also heated under the same conditions; no loss of their inhibitory ability was found. Thermal treatments have been used routinely for the inactivation of endogenous activities in inhibitor preparations from flour crude extracts (*18*−*20*); in fact, differently from other albumin proteins, their 3-D structures (9−10 cys residues in the sequence) makes these inhibitors very resistant to heat (*21*).

In-Gel α-Amylase Inhibitor Assay Optimization. Optimization of the starch-PAGE assay included the minimization of the amount of enzyme needed in the incubation buffer and

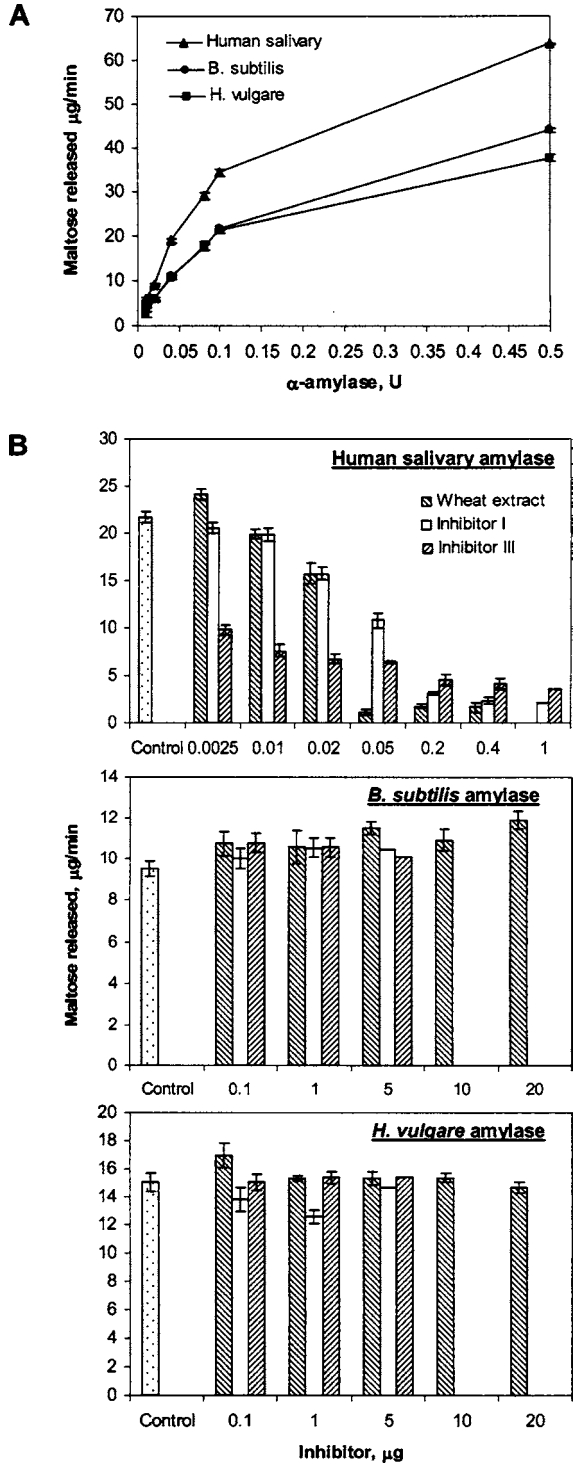

Figure 1. In-solution α-amylase and α-amylase inhibitor assay. (**A**) Commercial α-amylase activity curves from assays performed in 20 mM sodium phosphate buffer, pH 6.9, containing 6.7 mM sodium chloride. One unit of α-amylase was defined as the amount of enzyme that released 1.0 mg of maltose from starch in 1 min at 37 °C. Bars represent the standard error calculated for triplicate assays. The common value of 0.05 unit from the linear part of each curve was chosen as the optimal amount of enzyme to be used in the assays. (**B**) Inhibition of commercial α-amylase activities by the wheat flour extract and by the commercial α-amylase inhibitors I and III. The control sample contained no inhibitor. All assays were performed in triplicate, and the bars represent the estimated standard error.

improvement of protein band resolution. Given the general poor mobility of protein on Davis gels, the best results were obtained with the acrylamide concentration of 7.5% (**Figure 2**); lower concentrations were not even tested due to the possibility of starch leaking off the gel, excessive band vertical spreading, and difficult gel manipulation. Of the two starch concentrations (0.1 and 0.2%) tested, both worked well with our system, and the lower one was chosen to be copolymerized with the polyacrylamide. It is noteworthy that higher starch concentrations produced a widening of the protein bands, thereby strongly reducing their resolution (data not shown). Possibly, the poor protein resolution observed in the gels obtained by Giri and Kachole (*13*) was due to the higher level of incorporated starch.

The gels employed in the 1-DE assay method had a reduced thickness of 0.5 mm; due to the low acrylamide percentage, the gels were very flexible and elastic and could be quite easily manipulated without breaking. As for the amylase unit amounts to be employed in the assay, a minimum of 6 units per gel strip was needed for the complete digestion of the starch incorporated. A lower enzyme concentration left too dark a background, which did not contrast well with the undigested starch left where the inhibitor protein band localized. Reducing the amount of enzyme and compensating with a lengthened incubation time had an adverse effect on the procedure; in fact, no inhibitor protein band could be detected on gels incubated overnight, which otherwise would have given positive inhibition results. This was probably due to the poor ability of such a loose gel network to retain proteins, as suggested by the absence of protein bands on these gels when stained with CBB; the excessive digestion of the gel matrix starch could also contribute to this result.

When SDS was incorporated into the starch gels (**Figure 3**), the polyacrylamide concentration needed to be higher than for non-denaturing gels to avoid protein migration off the bottom. On the other hand, when the gel concentration was raised, protein migration became difficult, especially if the starch percentage was above 0.1. The best results with 0.1% starch were obtained on 12% polyacrylamide gels. Triton X-100 renaturation of proteins was found to be necessary for the recovery of the activity after electrophoresis separation under denaturing conditions (data not shown). Giri and Kachole (*13*) were not able to recover inhibitory amylase activities after SDS-containing gel treatment with Triton X-100. It is possible that the renaturation treatment failed due to the high starch concentration of their gels, which allowed for too strong starch−inhibitor complexes to form.

The application of the assay method to the revelation of inhibitor spots on 2-DE required the optimization of the IEF separation; non-denaturing conditions were necessary to maintain the integrity of the polymeric forms of some of the inhibitors and to detect all of the inhibitor forms. The wheat extract was dialyzed against water to keep at minimum the sample's salt concentration; after preliminary separations on IPG strips with a pH range 3−10, the final IEF separations were carried out in the pH range 5−8, which included all of the protein spots previously revealed. The second-dimension separation was found to be optimal when carried out with 0.75 mm 7.5% starch-PAGE gels.

In-Gel α-Amylase Inhibition Assay. Starch-PAGE gels were used to visualize the inhibitory activity of electrophoretically separated proteins (**Figure 2**); when the gels were incubated in an α-amylase solution, the starch incorporated was digested by the enzyme except in correspondence of an inhibitory protein

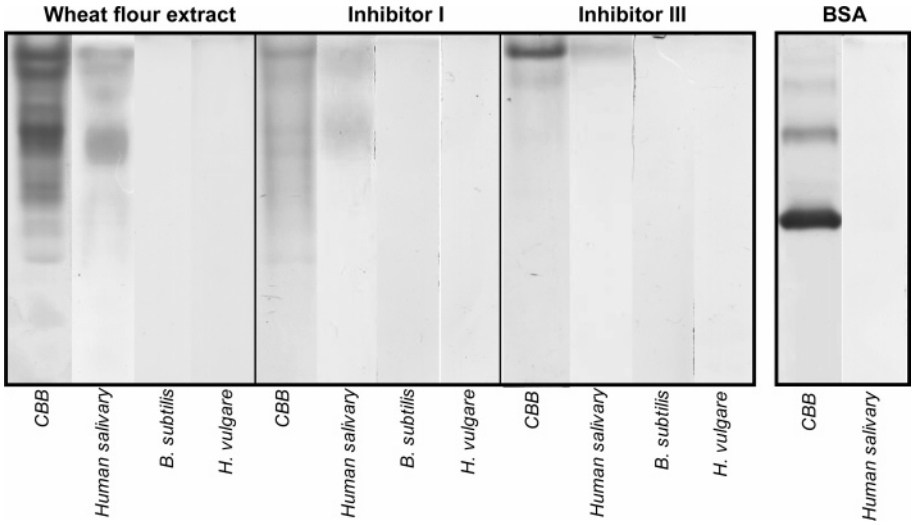

Figure 2. In-gel α-amylase inhibitor assay on 7.5% starch-PAGE; the gels incorporated 0.1% soluble starch. Each gel strip was incubated in 6 units of human salivary α-amylase for 1 h, at 37 °C. The strips were fixed in 10% TCA and stained with Lugol's reagent or with CBB R-350. Above the boxes are indicated the protein samples tested for inhibition of in-gel starch digestion. Below each gel slice is indicated the source of each α-amylase used for the gel incubation except for the slice labeled CBB, which was stained for proteins with CBB R-350 instead of being subjected to α-amylase treatment. Protein amounts applied: wheat flour extract, 31.5 μg; inhibitor I, 7.5 μg; inhibitor III, 5 μg; BSA, 5 μg.

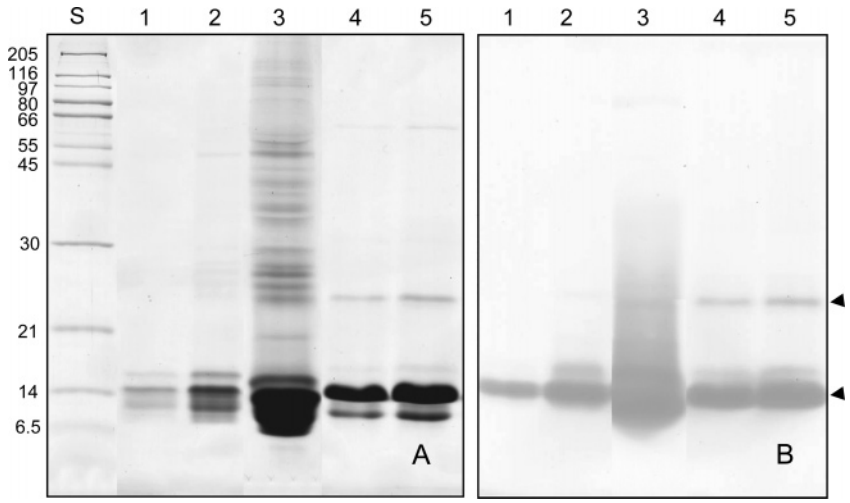

Figure 3. In-gel α-amylase inhibitor assay on 12% starch-PAGE containing 2% SDS: (**A**) gels stained for protein with CBB R-350; (**B**) gels stained for inhibitor visualization with Lugol's reagent. Each gel lane (strip) was incubated with 6 units of α-amylase for 1 h, at 37 °C. The strips were fixed in 10% TCA and stained. Lanes: 1–3, 1.25, 5, and 31.5 μg of flour extract, respectively; lanes 4 and 5, 5 and 10 μg of the commercial wheat inhibitor III, respectively; S, protein molecular weight standards, broad range (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Arrows point to the possible monomeric and dimeric forms of the inhibitors.

that impeded the enzyme action upon the starch. Afterward, when the gels were submerged with the Lugol's reagent, they stained dark blue/black only where the starch was still present. Thus, the inhibitor proteins resulted as the only protein bands stained against a clear, starch-digested, background. A useful tool to check for protein correspondence to the inhibitor band is the rapid destain (15−20 min) of the gels and restain with CBB, as already described by Martínez et al. (*16*). Due to the looseness of the gel network and despite the gel fixing with TCA, longer destaining times resulted in the loss of the faintest bands.

When optimized gels and assay conditions were used to separate and analyze the protein tested for α-amylase inhibition, the results obtained with the in-solution assays (**Figure 1B**) were confirmed by the in-gel assay (**Figure 2**, leftmost box); all of the inhibitors tested gave positive results against the human salivary amylase and had no effect on the other enzymes tested. BSA used as a negative control gave no inhibition response to the human salivary α-amylase (**Figure 2**, rightmost box).

Due to the polymeric nature of many of the wheat α-amylase inhibitors, the SDS-starch-PAGE assay (**Figure 3**) allowed for the revelation of mainly one protein band of about 12000 Da corresponding to the inhibitor monomeric component (**Figure 3B**); however, the chain separation was not complete—as shown in the figure—because a higher M_r protein (approximately 24000 Da) was revealed that also possessed inhibitory activity. However, the inhibitor's dimeric form was quantitatively less abundant than the monomeric, suggesting that many of the proteins were significantly denatured by the detergent. The presence of SDS in the electrophoresis sample buffer did not affect the inhibitor's polymeric nature any more than its presence

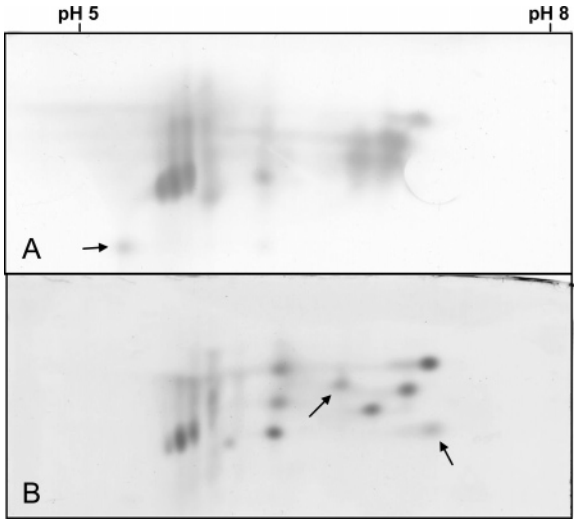

Figure 4. 2-DE of a flour extract. First-dimension separation was on 7 cm IPG strips, pH range 5–8; second-dimension separation was on 0.75 mm, 7.5% starch-PAGE gels: (**A**) 2-D gels stained with Lugol's reagent for inhibitor visualization. The inhibitory activity was developed as described in **Figure 2**, except that the gels were incubated in 20 mL of assay buffer containing 90 units of α-amylase. (**B**) 2-D gels stained with CBB R-350 for total protein visualization. Above the gels is indicated the pH range. The arrows point to protein spots unique to the respective gel. All gels were run in triplicate.

in the gel mixture and in the electrophoresis buffer (data not shown).

When increasing amounts of flour extract were applied on these gels (**Figure 3**), the inhibitory activity increased correspondingly; this would suggest an apparent linearity in the assay method response to varying inhibitor concentration.

Figure 4A shows the results obtained with the 2-DE assay of the flour extracts; several spots could be detected, representing the complex variety of the wheat kernel inhibitor isoforms. In fact, when duplicate gels were stained with Coomassie to detect all of the sample proteins and compared with the activity-stained ones, it became evident that most of them had inhibitory activity. Of all the spots detected, one could be revealed only with the activity staining (**Figure 4A**, arrow), indicating that the latter was more sensitive than the CBB R-350 staining. Only very few proteins seemed not to have an inhibitory activity (**Figure 4B**, arrows), further suggesting that the 50% ammonium sulfate precipitation and the boiling treatment of the flour extracts can be performed as useful prepurification steps for inhibitor preparations. Indeed, it has been shown that amylase inhibitors make up about two-thirds of the albumin of the wheat kernel (*22*).

In summary, we have developed an improved method for the electrophoretic detection of human salivary α-amylase inhibitory activity from cereal seeds extracts; we have shown that the heating pretreatment of the extracts is a necessary step for the appropriate inhibitor detection and that it does not impair their activity against α-amylase. Moreover, this treatment simplifies the isolation procedure, clearing the extract of most of the noninhibitory proteins. If SDS is included in the starch-PAGE, it is possible to evaluate the inhibitors' M_r, although mainly their monomeric forms can be recovered in the presence of detergent. By employing the newly developed 2-DE procedure in the starch-PAGE assay method, we were able to analyze the inhibitor multiple forms that are commonly found in cereal flours. This methodology seems therefore to be a promising tool for the proteomic analysis of food allergens and/or antinutrients and for the identification of new sources of plant resistance.

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