Detection of α-Amylase Activity in Unprocessed Preamylase Produced in the Cell-free Translation of Porcine Pancreatic RNA*

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Terry L. Brown† and Finn Wold
From the Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

Preamylases, synthesized in the RNA-dependent rabbit reticulocyte lysate translation system supplemented with porcine pancreatic RNA were identified by their specific immunoprecipitation with anti-amylase. The preamylases have apparent $M_r = 55,000$ and 58,000 as compared to 52,000 and 55,000 for the purified, secreted α-amylase isozymes. In order to establish whether the unprocessed precursors may assume enzymatically active conformations, we have explored a highly sensitive activity gel electrophoresis technique, by which picogram quantities of enzyme can be detected. When standard α-amylase and translation products are subjected to electrophoresis on polyacrylamide gel containing 0.01% starch and CaCl$_2$, active amylase which binds tightly to starch can only migrate as the starch is hydrolyzed. When the gel is subsequently stained with I$_2$, the appearance of clear tracks, the lengths of which are roughly proportional to the logarithm of amylase concentration, signifies the presence of amylase activity. By this approach, we were able to detect amylase activity in a range corresponding to about 100 pg of pure amylase/10 μl of translation mixture. This value agrees well with an estimate from radioactivity incorporation of total preamylase in the translation mixture, and we consequently conclude that unprocessed preamylase can assume the appropriate conformation to give enzymatic activity.

According to the signal hypothesis (1), secretory proteins are synthesized with an NH$_2$-terminal extension of 15 to 30 amino acids. These extra amino acids, the signal peptide or leader sequence, are involved in binding the polypeptides to the membrane of the endoplasmic reticulum and guiding the growing protein chain into the lumen of this organelle. The signal peptide is removed during the early stages of the polymerization process, presumably as the first of the many covalent and noncovalent modification steps that are involved in the processing of the genetically encoded polypeptide sequence into the finished secreted protein product. With recent successes in the cell-free synthesis of proteins, the question of whether the signal peptide removal is an obligatory first step in the production of biologically active proteins has become pertinent both as an essential piece of information and as a question that can be approached experimentally. Some information is available on this question as it relates to the in vitro synthesis of secreted enzymes. MacDonnel et al. (2) have stated that they were unable to obtain binding of precursor pancreatic amylase to glycogen, and Gorecki et al. (3) were unable to detect amylase activity in their translation mixtures containing the precursor form of parotid amylase. They discuss a possible physiological role for the synthesis of inactive precursor molecules and postulate that the signal peptide maintains the protein in an inactive form so that any of these digestive enzymes, improperly synthesized on free polysomes and subsequently released into the cytoplasm, would do no damage to the cell. However, Haugen and Heath (4) have shown that RNase precursor isolated by double-antibody precipitation and recovery of the enzyme under denaturing conditions yields a renatured precursor protein with activity in the range of native RNase. Therefore, at least in the case of RNase, the presence of the leader sequence itself does not prevent the denatured protein from folding into an active conformation.

In the course of our studies on the cell-free synthesis of pancreatic α-amylase, we have been able to assess the activity of the translation product by the use of a highly sensitive activity assay developed by Doane (5) which is based on substrate-containing acrylamide gel electrophoresis. The present communication describes the “activity-electrophoresis” assay system and reports the experimental basis for the conclusion that the unprocessed α-amylase precursor can assume a biologically active conformation.

MATERIALS AND METHODS

Isolation of Pancreatic RNA—Bovine and porcine pancreas were obtained from a slaughterhouse and immediately placed on ice. The RNA was isolated by the procedure of Ulrich et al. (6), in which the tissue is quickly homogenized in guanidine thiocyanate (Eastman Organic Chemicals) to denature the RNase present in large quantities in the pancreas of these species. The RNA was precipitated with ethanol and redissolved in guanidine HCl to remove protein and DNA. This step was repeated several times before finally dissolving the precipitate under ethanol and precipitating it with ethanol to remove traces of guanidinium ions, which interfere with protein synthesis. The RNA was stored at −20°C as a precipitate under ethanol to prevent degradation.

Cell-free Protein Synthesis—The pancreatic proteins were synthesized in the rabbit reticulocyte lysate system, using modifications of the procedures described by Hunt and Jackson (7) and Pelham and Jackson (8). Prior to use in the translation system, the lysate was made RNA dependent by treatment with micrococcal nuclelease (8). Lysate (0.5 ml) was treated with 12.5 μl of hemin (Sigma) solution (1 mM in 90% ethylene glycol, 20 mM Tris, pH 8.2, 50 mM KCl), 10 μl of micrococcal nucleases (15,000 units/ml), 5 μl of 0.1 M CaCl$_2$ and incubated at 20°C for 10 min. The nuclease digestion was terminated by the addition of 10 μl of 0.1 mM ethylene glycol bis(β-aminoethly ether)-N,N,N’,N”-tetraacetic acid.

The RNA-dependent translation system consisted of 40 μl of lysate, 25 μg of total pancreatic RNA, 25 μCi of [35S]methionine (1200 Ci/ mmol; New England Nuclear), 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate (Sigma), 5 μg of creatine phosphokinase (Sigma), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 100 mM KOAc, 1.2 mM Mg(OAc)$_2$, 6 mM β-mercaptoethanol, 4 μg of wheat germ RNA (Sigma), 500 μM spermidine, and the 19 amino acids at 200 μM (excluding methionine), in a total reaction volume of 100 μl. Translation reaction mixtures were incubated at 37°C for 90
min, then frozen for later studies.

Production of Anti-amylase Antibodies—Male New Zealand White rabbits (4 lbs) were given intramuscular injection of 1 mg of porcine amylase (Sigma) in Freund’s complete adjuvant. After 3 weeks, a second injection of 100 μg of porcine amylase in Freund’s incomplete adjuvant was given. After 10 days, blood was collected and the serum was sterilized by filtration through a Millipore filter (0.22 μm). Purification of the IgG fraction was achieved by passage through a DEAE–Affi-Gel blue column (Bio-Rad) in 0.02 M Tris–HCl, pH 8.0, containing 0.028 M NaCl and 0.02% NaN₃. Under these conditions, the IgG elutes from the column, leaving the majority of serum proteins and the proteolytic activity bound. Antibodies were analyzed for specificity by two-dimensional agar gel diffusion (9).

Immune Precipitation of Preamylase Synthesized in the Rabbit Reticulocyte Cell-free System—Translation products were analyzed for production of amylase precursor by incubation with anti-amylase antibody, and immune complexes were isolated by adsorption to Staphylococcus aureus cell walls obtained from Calbiochem (Pansorbin). Translation reaction mixtures (25 μl) were pretreated with 15 μl of a 10% suspension of SAC1 in 0.02 M phosphate, pH 7.6, 0.15 M NaCl, 0.25% Nonidet P-40 detergent (SAC buffer) for 15 min on ice to remove any components which bind nonspecifically to the S. aureus cell walls. The supernatant was collected by centrifugation for 2 min in an Eppendorf microfuge. The supernatant was then incubated with serum (20 μl of a 1:5 dilution) or the purified IgG fraction (1.5 μg) for 30 min at 4 °C to form antibody-antigen complexes. SAC (10 μl of 10% suspension) was added and incubated for 15 min at 4 °C to precipitate the immune complexes. The SAC and bound complexes were collected by centrifugation for 2 min and were washed 4 times by repeated resuspension and centrifugation with 0.5 ml of SAC buffer containing 5 mM methionine. The bound proteins were eluted from the SAC by incubation in 0.062 M Tris, pH 6.8, 3.5% SDS, 10% glycerol, 5% β-mercaptoethanol (sample application buffer for SDS gels) for 15 min at room temperature. Aliquots of this extract were analyzed by SDS gel electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis—Gels for size separation of translated proteins were prepared by the method of Laemmli (10), except that the separating gel consisted of a linear gradient of polyacrylamide from 10 to 14%. The gels were stained and dried, and autoradiography was performed using Kodak X-Omat RP film. If higher sensitivity was needed, stained gels were prepared for fluorography as described by Bonner and Laskey (11).

Activity Electrophoresis—The gels for the high sensitivity detection of amylase activity were prepared by modification of the system described by Ornstein (12) and Davis (13). These slab gels consisted of 7% acrylamide (Aldrich) cross-linked with 0.184 or 0.375% N,N,N’,N’-methylenebisacrylamide (Bio-Rad), respectively, and contained 0.37 M Tris–HCl, pH 8.9, 0.07% ammonium persulfate, 0.04% N,N,N’,N’-tetramethylethylenediamine, 5 mM CaCl₂ and 0.01% soluble starch, which had been boiled and centrifuged to remove insoluble material. The reservoir buffer contained 25 mM Tris, 5 mM CaCl₂, 0.19 M glycerol. The samples were applied in a solution of 40% sucrose, 5 mM CaCl₂, 0.005% bromophenol blue. The electrophoresis was conducted at a current of 15 mA for 5–6 h. The gels were then stained with a solution of 0.25% I₁ in 1% KI, which caused regions containing non-degraded starch to become grayish blue. Thus, any unstained, clear areas were regions of amylase activity, in which the starch had been digested.

RESULTS AND DISCUSSION

Preparation of Preamylase—Fig. 1A shows the products of cell-free synthesis in the rabbit reticulocyte lysate system using total porcine or bovine pancreatic RNA as template. In lane 1, the heaviest band (apparently a doublet) migrates slightly more slowly than the native porcine amylase isoforms, giving apparent M₅ ∼ 2000 to 3000 higher than the isolated pancreatic proteins. In the bovine system (lane 2), a much fainter band with the same mobility as the upper band of the doublet in the porcine system is assumed to be preamylase. Native bovine amylase migrates in this system with an apparent molecular weight corresponding to that of the upper band of the native porcine amylase. This suggests that in these species, amylase is synthesized as a preprotein containing a transient leader sequence, as is the case with most secretory proteins and as has been found for other amylases which have been studied (2, 3). The bands were identified as preamylase based on cross-reactivity to anti-amylase antibodies made against native porcine amylase, as shown in Fig. 1B. Lane 1 contains the products of translation with total porcine pancreatic RNA, and lane 2 contains translation products synthesized from bovine pancreatic RNA. B, immunochemical identification of preamylase. 1, total translation products synthesized from porcine RNA (as in A, lane 1); 2, fraction immunoprecipitated with anti-amylase and SAC; 3, fraction bound to SAC in the absence of anti-amylase.

FIG. 1. Autoradiogram (A) and fluorogram (B) of [³⁵S]methionine-labeled cell-free translation products from bovine and porcine pancreatic RNA separated by electrophoresis on 10–14% acrylamide gels containing SDS. A, molecular weight estimation. Positions of known protein markers (BSA, 67,000; ovalbumin, 43,000; deoxyribonuclease, 31,000; chymotrypsinogen, 25,000; ribonuclease, 13,600) and the two porcine α-amylase isoforms are indicated at left. Lane 1 contained translation products synthesized from porcine pancreatic RNA, and lane 2 contained translation products synthesized from bovine pancreatic RNA. B, immunochemical identification of preamylase. 1, total translation products synthesized from porcine RNA (as in A, lane 1); 2, fraction immunoprecipitated with anti-amylase and SAC; 3, fraction bound to SAC in the absence of anti-amylase.

1 The abbreviations used are: SAC, S. aureus cells; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.
duced in a typical experiment. Based on the specific activity of $[^{35}S] $methionine, number of methionines in the protein, amount of radioactivity in trichloroacetic acid-precipitated material, and ratio of amylase to total synthesized products (by gel densitometry of autoradiographs), a value of about 150 pg/10 μl of translation mixture is obtained.

Activity Electrophoresis—In our hands, the conventional α-amylase assay (15) did not have the needed sensitivity to detect the estimated quantities of enzyme potentially present in the translation mixture, and we consequently explored the activity electrophoresis technique developed and applied originally to DNase detection (16-18), but also extended to RNase (19, 20) and amylase (5). Fig. 2 illustrates typical results obtained with standard α-amylase and biological samples. In these starch-containing gels, amylase activity can be reliably detected down to the level of 50 pg. The enzyme is active during electrophoresis under the conditions used, so it degrades the starch in the region through which it travels, rather than displaying a band of activity at its final migration point. Since the enzyme binds tightly to the substrate, its mobility in the electrophoretic field will reflect the rate of hydrolysis of the starch in the gel matrix; in other words, the mobility will depend on the amount of starch and of active enzyme present. This is apparent in the almost linear relationship between mobility and the logarithm of enzyme concentration observed. We estimate that the lowest detectable level of amylase activity by this assay system is at least two orders of magnitude below that of the conventional amylase assays. As shown in Fig. 2, amylase activity could be detected at low levels in rabbit serum (lane 8). The amount corresponds roughly to the values given by Arnold and Rutter (21) for the level of amylase in rat serum. In lane 9, the purified IgG fraction (25 μg) also exhibits amylase activity at about 10% the level found in serum. This indicates that false positive results could be obtained upon assaying immune precipitates for amylase activity. There is also a slight indication of a cleared region when 5 μg of BSA (containing no amylase activity) is applied to the gel (lane 10). The staining of this region is very diffuse and readily distinguishable from the clear region observed when amylase activity is present. This artificial nonstaining effect is dependent on protein concentration, as we find that applying more BSA causes a larger although no more well defined semi-clear region. This is a condition of which to be aware when dealing with systems of very low specific activity. Careful controls must be run to ensure that amylase activity is being detected and artifacts are eliminated. It should also be pointed out that whereas the relative mobilities of standards and unknowns on different gels give consistent activity estimates, we have not been able to reproduce consistently the absolute mobilities from gel to gel.

When translation mixtures containing the synthesized amylase precursor protein were assayed by the activity electrophoresis technique, activity corresponding to an amylase concentration in the range of 100 pg/10 μl of translation mix was detected. In Fig. 3, 20 μl of translation products were applied to a starch-containing gel. Lanes 1 and 2 indicate the activity found in the translation products synthesized from porcine pancreatic RNA. Translation mixtures in which no synthesis of pancreatic proteins can occur (no RNA added) showed a slight diffuse clearance of the staining at the top of the track (lanes 3 and 4), which is very similar to the nonspecific protein effect discussed above. These lysates have a very high protein concentration, as demonstrated by the readily visible hemoglobin band which has migrated about one-third of the length into the gel. This difficulty is even more apparent when more translation mix is applied, as when attempting to study concentration effects. However, higher concentrations (50 μl) can definitely be shown to exhibit correspondingly higher levels of activity (data not shown). When proteins synthesized in

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**Fig. 2.** Detection of starch-hydrolyzing activity by activity gel electrophoresis on 7% acrylamide gel containing 0.5% soluble starch and 5 mM CaCl$_2$ according to the procedures given under "Materials and Methods." Lanes 1-7 contained (1) 5 μg, (2) 500 ng, (3) 50 ng, (4) 5 pg, (5) 500 pg, (6) 50 pg, and (7) 5 pg of standard α-amylase, respectively. Lanes 8-10 contained 5 μl of rabbit serum, 25 μg of purified rabbit IgG, and 5 μg of BSA, respectively, and lanes 11 and 12 contained sample application solution only.

**Fig. 3.** Detection of starch-hydrolyzing activity in cell-free translation products by activity gel electrophoresis under the same conditions as those described for Fig. 2. Lanes 1 and 2 contained 20 μl of translation products synthesized in the presence of porcine pancreatic RNA; lanes 3 and 4 contained the translation products from parallel controls from which the RNA had been omitted. Lane 5 contained sample application solution only.
translation mixtures containing bovine RNA were assayed for amylase activity, no difference can be seen over the no RNA controls. The level of amylase in bovine pancreas is less than 2% that found in porcine pancreas (22), and therefore should be below our limits of detection. Comparison of translation products synthesized from bovine or porcine RNA (Fig. 1A) shows that the level of preamylase synthesized is much greater in lysates supplemented with porcine RNA. From this evidence, we conclude that we can detect amylase activity in translation mixtures after synthesis of the unprocessed preamylase protein from porcine pancreatic RNA. The estimates of total preamylase in the translation mixture from radioactivity incorporation (150 pg/10 μl) and from activity measurements (100 pg/10 μl) are sufficiently close to suggest that the specific activity of preamylase could be approaching that of the native enzyme. It should be emphasized, however, that we do not believe that the activity electrophoresis technique can be used as a reliably quantitative assay and that both of the numerical values are very rough estimates at best. Nevertheless, the observation of any enzymatic activity in the preamylase sample, together with the demonstration of Haugen and Heath of enzymatic activity in preRNase (4), provides strong evidence that the removal of the signal peptide is not an obligatory step in the formation of active enzymes from all biosynthetic precursors.

In considering the overall processing of preamylase to active, secreted α-amylase, we visualize three distinct covalent chemical modification events: the proteolytic removal of the signal peptide, the acetylation of the NH₂-terminal (14), and the oxidation of the sulfhydryl groups to form disulfides. At some stage prior to the last of these events, the linear polypeptide must also have undergone the proper conformational adjustments to permit the correct pairing of sulfhydryl groups to take place. We had further visualized the most likely sequence of all these events in the biosynthesis of amylase would be 1) proteolysis, 2) acetylation, 3) conformational adjustments, and 4) disulfide formation. It now appears that step 1, and therefore presumably also step 2, is not required for steps 3 and 4 to proceed, and our model has been modified accordingly. We now consider it likely that both the signal peptide removal and the acetylation are relevant only to the secretion process, that the signal peptide portion of the preamylase sequence exists as an independent domain, quite separate from and without interaction with the remainder of the molecule, and consequently that steps 1 and 2 of the preamylase modification in principle can take place at any stage during the complete biosynthetic process. The role of the N-acetylation, which appears to be unique for amylase among the secreted pancreatic enzymes, and the requirement for and site of the presumed disulfide formation remain cryptic at this time.

We do not believe that the discrepancy between the previous reports concluding that preamylase is inactive (2, 3) and our conclusion that the precursor has amylase activity reflect subtle differences in spurious processing, such as disulfide formation, in the different preparations. On the basis of our own inability to demonstrate activity in the translation product by any of the conventional amylase assay methods, we feel confident that the apparently conflicting results simply reflect the greatly increased sensitivity of activity electrophoresis as an assay method.

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