Purification and characterization of α -amylase from rat pancreatic acinar carcinoma

Comparison with pancreatic α -amylase

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α -Amylase was purified to apparent homogeneity from normal pancreas and a transplantable pancreatic acinar carcinoma of the rat by affinity chromatography on α -glucohydrolase inhibitor (α -GHI) bound to aminoethyl-Sepharose 4B. Recovery was 95–100% for both pancreas and tumour α -amylases. They were monomeric proteins, with M_r approx. 54000 on SDS/polyacrylamide-gel electrophoresis. Isoelectric focusing of both normal and tumour α -amylases resolved each into two major isoenzymes, with pI 8.3 and 8.7. Tumour-derived α -amylase contained two additional minor isoenzymes, with pI 7.6 and 6.95 respectively. All four tumour isoenzymes demonstrated amylolytic activity when isoelectric-focused gels were treated with starch and stained with iodine. Two-dimensional electrophoresis, on SDS/10–20% -polyacrylamide-gradient gels after isoelectric focusing, separated each major isoenzyme into doublets of similar M_r values. Pancreatic and tumour-derived α -amylases had similar K_m and K_i (α -GHI) values, but the specific activity of the tumour α -amylase was approximately two-thirds that of the normal α -amylase. Although amino acid analysis and peptide mapping with the use of CNBr, *N*-chlorosuccinimide or *Staphylococcus aureus* V8 proteinase gave comparable profiles for the two α -amylases, tryptic-digest fingerprint patterns were different. Antibodies raised against the purified pancreatic α -amylase and tumour α -amylase respectively showed only one positive band on immunoblotting after gel electrophoresis of crude extracts of rat pancreas and carcinoma, at the same position as that of the purified enzyme. More than 95% of the α -amylase activity in the pancreas and in the tumour was absorbed by an excess amount of either antibody, indicating that normal and tumour α -amylases are immunologically identical. The presence of additional isoenzymes in the carcinoma, and dissimilarity of tryptic-digest patterns, may reflect an alteration in gene expression or in the post-translational modification of this protein in this heterogeneously differentiated transplantable pancreatic acinar carcinoma.

INTRODUCTION

The pancreatic enzyme α -amylase (α -1,4-glucan glucanohydrolase, EC 3.2.1.1) has been extensively studied in a number of species, including rat, mouse, rabbit, man, dog and guinea pig (Malacinski & Rutter, 1969; Hagenbüchle *et al.*, 1981). The existence of several electrophoretic and immunological variants of α -amylase has been established by numerous investigators. For example, α -amylase is a major constituent of pancreatic and parotid secretions, and is also synthesized and secreted in small quantities by the liver (Arnold & Rutter, 1963; McGeachin *et al.*, 1964; Messer & Dean, 1975; Takeuchi *et al.*, 1975), but these enzymes exhibit differences in peptide composition. Differences in antigenic properties between pancreatic α -amylase, parotid α -amylase and liver α -amylase in some animals have also been noted (Sanders & Rutter, 1972; Stiefel & Keller, 1973). Furthermore, rat pancreatic α -amylase mRNA is distinct from that of liver and parotid, indicating the presence of at least two α -amylase genes (Harding & Rutter, 1978; Hagenbüchle *et al.*, 1980, 1981). The existence of isoenzymes and the relative ease of detection and purification of α -amylase make this

enzyme an ideal marker for the study of control mechanisms for differential gene expression in pancreatic-acinar-cell neoplasia.

A transplantable rat pancreatic acinar carcinoma (PACA) established in F344 rats by Reddy & Rao (1977) has been shown to synthesize amylase along with other exocrine pancreatic enzymes (J. K. Reddy *et al.*, 1980a; Hansen *et al.*, 1983). Just as the normal pancreas provides an excellent model for the study of cytodifferentiation and gene expression, this tumour serves as a model for the study of altered gene expression in neoplasia. The carcinoma displays heterogeneous cytodifferentiation, containing cells ranging from those with numerous zymogen granules, with a morphology resembling that of the normal pancreatic exocrine cell, to those which are undifferentiated and lack well-formed secretory (zymogen) granules (J. K. Reddy *et al.*, 1980b; Becich & Reddy, 1982). α -Amylase is detectable enzymically and by the Protein A–gold immunocytological method in neoplastic cells: the values, however, are considerably higher in the well-differentiated secretory-granule-enriched cells than in secretory-granule-deficient undifferentiated tumour cells (Bendayan *et al.*, 1984).

In the present paper we have compared the biochemi-

Abbreviations used: α -GHI, α -glucohydrolase inhibitor; AH-Sepharose 4B, 2-aminoethyl-Sepharose 4B; PACA, transplantable pancreatic acinar carcinoma.

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cal, molecular and immunological properties of rat pancreatic-carcinoma α -amylase with normal rat pancreatic α -amylase in order to determine alterations, if any, occurring as a result of neoplastic transformation in this differentiation-related pancreas-specific protein.

MATERIALS AND METHODS

Materials

Acrylamide, *NN'*-methylenebisacrylamide and *NNN'*-tetramethylethylenediamine were purchased from Eastman Kodak, Rochester, NY, U.S.A. Coomassie Brilliant Blue R, phenylmethanesulphonyl fluoride, EDTA, soya-bean trypsin inhibitor, NH_4HCO_3 , CNBr and *N*-chlorosuccinimide were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Ammonium persulphate, M_r standards and SDS were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Trypsin [242 units/mg; treated with 1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')] was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Ampholines (pH 3.5–10, pH 9–11, pH 4–6 and pH 5–7) were obtained from LKB, Uppsala, Sweden. α -GHI (acarbose or Bay G5421) was a gift from Miles Laboratories, Elkhart, IN, U.S.A. AH-Sepharose 4B was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.

Rat pancreatic acinar carcinoma

For these studies a pancreatic acinar carcinoma established in F344 rats as a transplantable tumour by Reddy & Rao (1977) was used. Tumour transplantation was done either subcutaneously or intraperitoneally in weanling F344 male rats (weighing 40–60 g) as previously reported (J. K. Reddy *et al.*, 1980b). All tumours used in this study were between the 35th and 50th passage. Normal pancreas was obtained from adult F344 male rats, weighing about 250–300 g.

Binding of affinity ligand α -GHI to AH-Sepharose 4B for affinity chromatography of α -amylase

α -GHI was covalently bound to AH-Sepharose 4B (Burrill *et al.*, 1981). AH-Sepharose 4B (4 g) was washed and equilibrated with 0.2 M-sodium phosphate buffer, pH 7.0. α -GHI (200 mg) was added and mixed at 4 °C for 30 min, followed by 100 mg of NaBH_4 , which was left to react for 6 h at 4 °C with continuous shaking. An additional 100 mg of NaBH_4 was added to the mixture and left to react at 4 °C for 22 h. The gel was then washed with double-distilled water and packed in a column (1 cm $\times$ 10 cm). The column was equilibrated with 0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4.

Purification of α -amylases from normal pancreas and PACA

Male F344 rats weighing 250–300 g were killed under light diethyl ether anaesthesia, and the pancreata were removed, trimmed free of fat and kept frozen until used. PACA tissue from the tumour-bearing rats was removed and kept frozen for the purification of α -amylase. Tissues were homogenized in 9 vol. of 0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-phenylmethanesulphonyl fluoride at 4 °C in a Potter-Elvehjem motorized tissue grinder. Homogenates were centrifuged at 105 000 *g* for 30 min in a Ti50 rotor

at 2 °C. Samples (about 10 ml) of the supernatants were applied to the affinity column at a flow rate of 0.25 ml/min. After the sample moved into the column, the flow was stopped for 30 min. The sample was washed with sample buffer (0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-phenylmethanesulphonyl fluoride until all unbound protein was washed out. α -Amylase activity was eluted by washing the column with 0.1% glycogen in the sample buffer. All protein showing α -amylase activity was pooled and made 40% (v/v) with respect to ethanol containing 1 mg of glycogen (shellfish)/ml; the mixture was centrifuged at 12 000 *g* for 1 h to collect the precipitate. The precipitate was suspended in sample buffer containing 5 mM- CaCl_2 and incubated at room temperature for 2 h. Denatured protein and glycogen were removed by centrifugation at 10 000 *g* for 10 min (Loyter & Schramm, 1962). The supernatant was used for various analyses reported below. Dextran-free α -amylase was prepared by passing the α -amylase sample through a column of charcoal and Celite as reported by Sanders & Rutter (1972). Enzymic activity of the purified protein separated on an isoelectric-focusing gel was demonstrated by staining with the KI/I₂ method of Malacinski & Rutter (1969).

Assays

α -Amylase activity was measured as the rate of conversion of starch into maltose by the method of Stein & Fisher (1958). Protein content was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, or by u.v. absorption by using the absorption coefficient $A_{1\%}^{1\text{cm}, 280} = 20$. The substrate and inhibition constants were determined from Lineweaver-Burk plots by the method described by Sanders & Rutter (1972).

Preparation of antibodies

Antibodies against α -amylase purified from normal rat pancreas and PACA were generated in New Zealand White male rabbits as reported previously (M. K. Reddy *et al.*, 1980). The antisera were stored, at -20 °C. Immunodiffusion was done in 1% agar gel as described by Ouchterlony (1977), and Western blotting was done by the method of Towbin *et al.* (1979).

Isoelectric focusing

Isoelectric focusing of purified α -amylase was performed at 3.5–10.0 (Hansen *et al.*, 1983) as described by Scheele (1975). SDS/polyacrylamide-gel electrophoresis was done in slab gels by the method of Laemmli (1970) as described previously (M. K. Reddy *et al.*, 1980). The gels were stained with Coomassie Blue.

Peptide mapping of α -amylase by enzymic cleavage with *Staphylococcus aureus* V8 proteinase was done as described by Cleveland *et al.* (1977) on an SDS/10–20% polyacrylamide-gradient gel. Peptide mapping of α -amylase by chemical cleavage was done with *N*-chlorosuccinimide/urea reagent as cleaving agent with an SDS/15% polyacrylamide gel (Lischwe & Ochs, 1982), or with CNBr as described previously (Nikodem & Fresco, 1979). Tryptic-peptide digests of carboxymethylamido- α -amylase were analysed on Whatman 3MM paper (Ritschard, 1964). Electrophoresis was done in pyridine/acetic acid/butanol/water (1:1:2:32, by vol.), pH 6.5, maintained at 20 °C, at 2000 V for 1 h. Paper chromatography was performed in butanol/acetic acid/water (4:1:5, by vol.).

Fig. 1. α -GHI-AH-Sepharose 4B column chromatography of rat PACA α -amylase

A 10% extract of acinar cell carcinoma (120 mg of protein; approx. 2000 units of activity) in 0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-phenylmethanesulphonyl fluoride was loaded on a column (1 cm $\times$ 10 cm) of α -GHI-AH-Sepharose 4B, equilibrated with the same buffer as explained in the Materials and methods section. Protein was eluted with 0.1% glycogen in 0.15 M-NaCl/5 mM-sodium phosphate buffer, pH 7.4; 3 ml fractions were collected and analysed. ●, A_{280} ; ○, α -amylase specific activity. Arrow indicates the start of elution with glycogen.

RESULTS

Purification of and catalytic properties of normal pancreatic and PACA α -amylases

α -Amylases from normal rat pancreas and PACA were each purified to homogeneity by a single-step procedure using affinity chromatography on α -GHI-AH-Sepharose 4B. Fig. 1 represents the typical elution profile of α -amylase from the affinity column. α -Amylase was eluted as a single symmetrical peak with a constant specific enzyme activity; 99% of the original enzyme activity in the 105000 g supernatant was recovered under these conditions. Similar results were obtained with normal pancreatic extracts. The specific activity of the enzyme in both cases increased over 30-fold (Table 1).

Fig. 2. SDS/polyacrylamide-gel electrophoretograms of α -amylases purified by affinity chromatography from normal pancreas (lane 1; approx. 20 μ g of protein) and PACA (lane 2; approx. 10 μ g of protein)

SDS/10% (w/v)-polyacrylamide gels after electrophoresis were stained with Coomassie Brilliant Blue. Positions of Bio-Rad low- M_r standards are shown on the left. The α -amylases had M_r approx. 54000.

Enzyme specific activity and the yield of α -amylase per g of tissue from PACA was approx. 36% lower than that of normal pancreatic amylase. A single polypeptide chain with M_r 54000 was found for both proteins (Fig. 2). Chromatography on Sephadex G-100 column gave an estimate of M_r 54000 with bovine serum albumin, ovalbumin and creatine kinase as standards (results not shown). Table 2 summarizes some catalytic properties of α -amylases obtained from rat pancreas and PACA. The enzymic activity of α -amylase in the serum of carcinoma-bearing rats increased approx. 6-fold as compared with normal rat serum. However, the activity (units/g of tissue) and the specific activity of both crude and purified α -amylase from PACA were 36–38% less than for the normal α -amylase. Both α -amylases showed the same pH

Table 1. Purification of α -amylase by affinity chromatography on α -GHI-AH-Sepharose 4B

Activity is expressed as μ mol of maltose produced/min at 37 °C.

Source	Specific enzyme activity (μ mol/min per mg of protein)		Recovery (%)	Purification (fold)	Content of pure enzyme (mg/g of tissue)
	Before chromatography	After chromatography			
Normal pancreas	31.7 $\pm$ 3.3	1018 $\pm$ 10	100	32.2	7.05 $\pm$ 0.05
PACA	21.4 $\pm$ 5.5	654 $\pm$ 8	99	30.6	3.90 $\pm$ 0.10

Table 2. Comparative analysis of α -amylases from normal pancreas and PACA of F-344 rats

Parameter	Normal pancreas	PACA
Enzyme activity		
Serum (units/ml)	453 $\pm$ 225*	2572 $\pm$ 719
Tissue		
Total (units/g)	6178 $\pm$ 235†	3833 $\pm$ 872
Specific (units/mg of protein)	31.7 $\pm$ 3.3	21.4 $\pm$ 5.5
Affinity-purified (units/mg of protein)	1018 $\pm$ 108	654 $\pm$ 80
M_r on SDS/polyacrylamide-gel electrophoresis	54000	54000
Isoelectric focusing		
No. of isoenzymes	2	4
pI values	8.3	8.7 8.3 7.6 6.95 (very low activity; often difficult to identify by the demonstration of enzyme activity)
Optimal pH	6.9	6.9
Optimal [Ca ²⁺]	5 mM	5 mM
K_m (α -GHI)	2.5 μ g/ml	2.5 μ g/ml
K_i (α -GHI)	4.0 μ g/ml	4.0 μ g/ml

* Mean $\pm$ S.D. for three animals.† Mean $\pm$ S.D. for five animals.

optimum (6.9), the same Ca²⁺ requirements for enzyme activation (5 mM), the same K_m (2.5 mg/ml, with starch as substrate) and the same K_i (Table 2) (4 μ g of α -GHI/ml, with α -GHI as inhibitor). However, they differ in V_{max} and the number of isoenzymes (Table 2).

Molecular properties of normal pancreatic and PACA α -amylases

Isoelectric focusing in polyacrylamide gel separated purified α -amylase from normal pancreas into two bands, with pI 8.7 and 8.3 (Fig. 3b). PACA α -amylase separated into three bands, with pI 8.7, 8.3 and 7.6 (Fig. 3b, Coomassie-Blue-stained). Occasionally a faint fourth band, with pI 6.95, was observed (barely visible in the Coomassie-Blue-stained gel; see Fig. 3b). To detect all four bands with the KI/I₂ method, either the quantity of enzyme or the incubation time had to be increased severalfold. All the bands possessed demonstrable enzyme activity (Fig. 3a). The α -amylase isolated as an α -amylase-glycogen complex, however, possessed only two isoenzymes (pI 8.7 and 8.3), whether isolated from the normal or the neoplastic pancreas.

To investigate the structural differences between normal pancreatic α -amylase and PACA α -amylase, we have analysed the peptides generated by *Staph. aureus* V8 proteinase, by gel electrophoresis. The SDS/polyacrylamide-gel-electrophoretic profile of the peptides produced by the proteinase treatment showed no differences in the patterns, suggesting similar structure for both proteins (results not shown). Analysis of the cleavage fragments of α -amylase produced by *N*-chlorosuccinimide in the presence of urea, which results in cleavage at tryptophyl peptide bonds (Lischwe & Ochs, 1982), also showed identical peptide maps for the

Fig. 3. Isoelectric focusing (pH 3.5–10) in a polyacrylamide slab gel of purified α -amylases from normal pancreas (N) and PACA (C)

(a) Demonstration of enzymic activity by staining with KI/I₂; (b) gels stained with Coomassie Brilliant Blue.

Table 3. Amino acid analysis of α -amylases from normal pancreas and PACA

Amino acid	Content (mol/54000 g of protein)		
	Normal pancreatic α -amylase	PACA α -amylase	Rat pancreatic α -amylase*
Asx	81	81	85.7
Thr	23	23	21.5
Ser	34	34	29.0
Glx	39	39	30.8
Pro	22	20	19.4
Gly	58	57	50.9
Ala	37	37	34.6
Cys	10	10	11.1
Val	34	36	36.9
Met	9	9	10.6
Ile	23	23	26.6
Leu	24	24	24.6
Tyr	15	15	16.5
Phe	21	21	24.7
Lys	29	29	23.9
His	14	13	13.0
Arg	27	27	27.2
Total	500	498	487.0

* Vandermeers & Christophe (1968).

α -amylases from normal pancreas and from PACA (results not shown).

Amino acid compositions of normal pancreas and PACA α -amylases are given in Table 3. A high degree of similarity of amino acid composition is readily seen. A

total of 50 lysine and arginine residues is found in α -amylases from normal pancreas and PACA; hence a complete digestion with trypsin would yield approx. 50 peptides for a single-subunit enzyme. Peptide maps of these two enzymes are given in Fig. 4; approx. 50 peptides were derived from each protein. The pattern of peptide maps obtained for α -amylase from normal pancreas was slightly different from that of the PACA enzyme.

Immunological properties of pancreatic and PACA α -amylases

The antibodies produced in rabbits against normal and PACA α -amylases were monospecific. The antigen-antibody complexes retained the enzymic activity. Activity remaining in the supernatants after immunotitration approached zero with increasing quantity of antibody. Similar results were obtained with either anti-(PACA α -amylase) or anti-(normal α -amylase) antibodies (results not shown). Ouchterlony double-diffusion tests showed a single precipitin line with normal pancreatic extract or PACA extract, with anti-normal α -amylase antibody (Fig. 5a). Similar immunodiffusion bands were obtained with purified enzymes (results not shown.) Fig. 5(b) presents the immunodiffusion pattern obtained with antiserum against PACA α -amylase. Immunoblotting with anti-(normal α -amylase) or anti-(PACA α -amylase) antibody showed similar patterns (Fig. 6). The normal pancreas has more α -amylase content than does PACA. Similar values were obtained during the purification procedure (Table 2). The immunological properties of both pancreatic and PACA α -amylases were identical, as observed from immunodiffusion, immunoblotting and immunoinhibition studies.

Fig. 4. Fingerprinting of normal pancreatic and PACA α -amylase

Tryptic digests were separated on Whatman 3MM paper. Electrophoresis was at 20 °C for 1 h at 2000 V, in pyridine/acetic acid/butanol/water (1:1:2:32, by vol.). Chromatography was in butanol/acetic acid/water (4:1:5, by vol.) and the peptides were identified as ninhydrin-positive spots.

Fig. 5. Ouchterlony double-immunodiffusion pattern of anti-(pancreatic α -amylase) and anti-(PACA α -amylase) against (a) purified α -amylases or (b) extracts of α -amylases from normal pancreases and PACA

Well 1, normal pancreas extract; well 2, carcinoma extract; well 3, antiserum; wells 4–8, 1 μ g, 2 μ g, 3 μ g, 5 μ g and 10 μ g of purified α -amylase respectively; well 9, 0.9% NaCl.

Fig. 6. Western blots of extracts of normal rat pancreas (a) and PACA (b) with anti-(pancreatic α -amylase) serum

Known quantities of high-speed tissue extracts were separated by electrophoresis on an SDS/10%-polyacrylamide gel and were transferred to nitrocellulose paper. The paper was treated with anti-(pancreatic α -amylase) serum (diluted 1:50). The resulting immunocomplex was identified by treatment with 125 I-labelled protein A by autoradiography. Lanes 1, 2 and 3 contained 5, 10 and 25 μ g of protein of the extract respectively, and lane 4 contained either 5 μ g (a) or 2 μ g (b) of purified α -amylase respectively.

DISCUSSION

The pancreatic carcinoma established as a transplantable tumour by Reddy & Rao (1977) is a useful model for the studies of altered cytodifferentiation and gene expression in a neoplastic tissue. The secretory proteins synthesized by this tumour are products normally associated with the highly specialized well-differentiated adult pancreatic exocrine cell. It is therefore of interest to determine how the expression of such proteins is regulated in the neoplastic state.

We have purified α -amylases from this pancreatic acinar carcinoma and from normal F-344 rat pancreas. The affinity-chromatography procedure of Burrill *et al.* (1981) yielded highly purified homogeneous fractions of α -amylase from both tissues as a single homogeneous protein peak. No proteolytic degradation or contaminating proteins were detectable on Coomassie-Blue-stained SDS/polyacrylamide-gel electrophoretograms.

Having established the purity and integrity of the enzyme preparations, various properties of the normal and tumour-derived enzymes were compared. Both normal pancreatic and tumour α -amylases contained two isoenzymes, pI 8.3 and 8.7, on isoelectric-focusing gels. However, two additional but faint isoenzymes, pI 7.6 and 6.95, were also detectable in tumour α -amylase isoelectric-focusing gels. These two isoenzymes were not detected in tumour α -amylase purified by the method of Loyter & Schramm (1962), using glycogen precipitation, which may suggest higher affinity for the α -GHI than for glycogen as a substrate at low concentrations of the enzyme.

Enzyme kinetic data showed distinct differences between tumour and normal α -amylases. Although K_m (starch) and K_i (α -GHI) were identical, the specific activity of the tumour α -amylase was significantly decreased relative to the normal pancreatic enzyme.

Several methods of analysis indicate that, despite some differences in enzymic properties between tumour and normal α -amylases, considerable structural homology is retained. The two enzymes are immunologically identical, as determined by Ouchterlony double-diffusion, immunoblotting and immunoinhibition studies performed with antibodies raised against both α -amylases. Amino acid analysis of the two enzymes also showed striking similarity. The minor differences observed, and the differences from the data of Vandermeers & Christophe (1968), are generally within the experimental error of the technique. Although these data did not distinguish between compositions of isoenzyme, previously reported data indicate only minor differences among isoenzymes of the same species and, in some cases, of different species.

Although peptide mapping with the use of CNBr, *N*-chlorosuccinimide or *Staph. aureus* V8 proteinase as cleaving agents yielded essentially similar patterns, tryptic-digest fingerprints showed significant differences. Thus the primary structure of PACA α -amylase is different from that of normal α -amylase, despite the similar amino acid composition.

From these data we conclude that normal rat pancreatic and PACA α -amylases constitute closely related molecules. The results do not distinguish among the isoenzymes and represent a composite of their properties; further work to elucidate their individual properties is necessary, particularly to characterize the

molecular properties of the two additional isoenzymes seen in tumour α -amylase. The nature of the genetic basis of these alterations also remains to be clarified. Harding & Rutter (1978) have shown the existence of several α -amylase genes within the rat genome, and expression of a new gene could be responsible for the changes seen in this tumour. These changes may also be due to neoplasia-induced epigenetic mechanisms. Hagenbüchle *et al.* (1980, 1981) have described a single mouse α -amylase gene that codes for two different tissue-specific mRNAs in normal tissue. The subtle changes observed here may therefore be due to transcriptional or post-transcriptional modifications in the expression of the normal rat pancreatic α -amylase genes, or to changes in glycosylation in tumour cells (Bernacki *et al.* 1985). Additional studies are necessary to determine whether the multiple forms of α -amylase seen in PACA are due to possible alterations in acetylation of the *N*-terminus or to deamidation resulting from neoplastic transformation.

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