Preparative isolation of the two forms of pig pancreatic pro-(carboxypeptidase A) and their monomeric carboxypeptidases A

Maria VILANOVA, Josep VENDRELL, M. Teresa LÓPEZ, Claudi M. CUCHILLO
and Francesc X. AVILÉS*

*Departament de Bioquímica, Facultat de Ciències i Institut de Biologia Fonamental 'Vicent Villar Palasi',
Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain*

(Received 17 December 1984/11 March 1985; accepted 15 April 1985)

A method is reported for the preparative isolation of the two forms of pro-(carboxypeptidase A) from pig pancreas: the monomer and the binary complex with pro-(proteinase E). This method, which is mainly based on chromatography on DEAE-Sepharose at pH 5.7, allows these proenzymes to be prepared more quickly and in safer conditions than with other reported methods. Undegraded and homogeneous carboxypeptidase A₁ and A₂ species (peptidyl-L-amino acid hydrolase, EC 3.4.17.1), in monomeric forms with high specific activity, are also obtained in high yield by controlled trypsin activation of either of the pro-(carboxypeptidases A) followed by chromatography on DEAE-Sepharose at pH 5.8 under dissociating conditions (7M-urea).

The preparative isolation of native pro-carboxypeptidases and other digestive proenzymes from mammalian pancreatic tissue is not an easy task. This tissue contains about a dozen different proteinases (in the zymogen form) (Scheele, 1975), with a high potentiality for activating and degrading each other. The development of new methods for a quicker isolation of the above proenzymes, decreasing the number of purification steps and improving safety conditions, can therefore be of great value.

Pro-(carboxypeptidase A) occurs in pig pancreas in two different forms, as a monomer (*M_r* 45000) and as a stable binary complex with pro-(proteinase E) (*M_r* 45000+26000) (Kobayashi *et al.*, 1978; Martínez *et al.*, 1981). The coexistence of both forms provides a good system for the structural and functional characterization of this class of proenzymes, as mentioned previously (Martínez *et al.*, 1981; San Segundo *et al.*, 1982; Vendrell *et al.*, 1982). Thus in this system the effect of complex-formation on the activation process and the different stages that take place in this process can readily be studied at the molecular level.

However, the isolation of the two pig pro-(carboxypeptidases A) by the methods used currently (Folk & Schirmer, 1965; Kobayashi *et al.*, 1978; Martínez *et al.*, 1981) is a long and tedious

task (usually 2 weeks), with a high risk of degradations. In the present paper we now describe a preparative method that allows a faster separation of both zymogen forms (in only 4 days) under safer conditions. In this paper we also describe a method that, starting from either of the two pro-(carboxypeptidases A), produces in a controlled way active monomeric carboxypeptidase A₁ and A₂ forms, without degradation and with high activity.

Materials and methods

Detection and quantification of pancreatic pro-proteinases was carried out by spectrophotometric measurements of enzymic activities (before and after tryptic activation) on artificial substrates: carboxypeptidase A and carboxypeptidase B on benzoylglycyl-L-phenylalanine and benzoylglycyl-L-arginine respectively (Folk & Schirmer, 1963); chymotrypsin on *N*-acetyl-L-tyrosine ethyl ester (Schwert & Takenaka, 1955). Electrophoresis was carried out in 0.1% sodium dodecyl sulphate/polyacrylamide gradient gels (12–19% acrylamide) by the method of Laemmli (1970), or in 7M-urea/polyacrylamide gels (9.5% acrylamide) by the method of Creighton (1979). Automated amino acid analyses were performed by the method of Moore & Stein (1963).

For the isolation of pro-carboxypeptidases, 100g

* To whom correspondence should be addressed.

of defatted acetone-dried powders from pig pancreas were suspended in 1 litre of distilled water, and the pancreatic proteins were extracted and subsequently fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, by the method of Folk & Schirmer (1963). The precipitated pro-(carboxypeptidases A) and pro-(carboxypeptidase B) were redissolved in 40 mM-Tris/HCl buffer, pH 8.0, and, after overnight dialysis against this buffer, were chromatographed on a column (3.5 cm $\times$ 50 cm) of DEAE-Sephacel CL-6B (Pharmacia) equilibrated with the same buffer. The chromatography was developed with a linear salt gradient of 0–0.45 M-NaCl. Fractions containing a high carboxypeptidase A potential activity (Fig. 1) were pooled and concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 65% saturation.

To achieve the separation of the two forms of pro-(carboxypeptidase A), the last precipitate was redissolved in 40 mM-Tris/acetate buffer, pH 5.7, containing 0.1 M-NaCl, and, after overnight dialysis against this buffer, the solution was chromatographed on a column (3.5 cm $\times$ 50 cm) of DEAE-Sephacel CL-6B equilibrated with the same buffer. The proteins were eluted with a linear salt gradient of 0.1–0.35 M-NaCl (Fig. 2). To each

fraction containing only one form of pro-(carboxypeptidases A), phenylmethanesulphonyl fluoride and $(\text{NH}_4)_2\text{SO}_4$ were added so as to reach concentrations of 1 mM and 65% saturation respectively. The samples were stored at 4°C. It should be noted that soya-bean trypsin inhibitor at a concentration of 0.1 mg/ml was added to each solution containing pro-(carboxypeptidase A) at the beginning of their isolation and at each subsequent step. All operations were carried out at 4°C.

In order to obtain monomeric carboxypeptidases A, 100 mg of pro-(carboxypeptidase A) binary complex (or 65 mg of the monomeric form) at 110 μM in 20 mM-sodium phosphate buffer, pH 5.8, was treated with bovine trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one-('TPCK')-treated; Worthington Biochemical Corp.] at 5.5 μM for 1 h at 25°C. After the addition of 15 mg of soya-bean trypsin inhibitor, the digest was loaded on to a DEAE-Sephacel CL-6B column (1.5 cm $\times$ 45 cm) equilibrated with 7 M-urea/50 mM-sodium phosphate buffer, pH 5.8. Active proteinase E, the two different monomeric carboxypeptidase A species, the corresponding activation peptides and the added trypsin inhibitor were sequentially eluted from the column by the

Fig. 1. Chromatography of pig pancreatic proteins on DEAE-Sephacel at pH 8.0

Proteins from the aqueous extract of pancreatic acetone-dried powder were submitted to a differential precipitation with $(\text{NH}_4)_2\text{SO}_4$, redissolved, dialysed and loaded on to a DEAE-Sephacel column equilibrated with 40 mM-Tris/HCl buffer, pH 8.0, and eluted with a linear gradient of 0–0.45 M-NaCl (.....). The absorbance at 280 nm was monitored (—), and potential and free activities of the individual fractions were measured against *N*-acetyl-L-tyrosine ethyl ester for chymotrypsinogens ($\blacktriangle$ — $\blacktriangle$ and $\triangle$ — $\triangle$); benzoylglycyl-L-phenylalanine for pro-(carboxypeptidases A) ($\bullet$ — $\bullet$ and $\circ$ — $\circ$), and benzoylglycyl-L-arginine for pro-(carboxypeptidases B) ($\blacksquare$ — $\blacksquare$ and $\square$ — $\square$).

application of two successive linear salt gradients of 0–70 mM-NaCl and 70 mM–1 M-NaCl (see Fig. 3), both in 7 M-urea/50 mM-sodium phosphate buffer, pH 5.8.

Results and discussion

Isolation of pro-(carboxypeptidases A)

The preparative isolation of the two forms of pig pancreatic pro-(carboxypeptidases A) described in the Materials and methods section is based on three main steps: (i) an initial fractionation by salting out; (ii) a chromatography on DEAE-Sephacrose at pH 8.0; (iii) a second DEAE-Sephacrose chromatography at pH 5.7. After the first chromatography at pH 8.0, carboxypeptidase B, carboxypeptidase A, pro-(carboxypeptidase A), pro-(carboxypeptidase B) and other pancreatic proteins are obtained as major components of seven different chromatographic fractions. This is shown by analysis of free and potential activities along the chromatographic profile and by electrophoresis of the fractions (Figs. 1 and 2). None of these fractions contains a pure and homogeneous

protein. The two forms of pro-(carboxypeptidase A), together with pro-(carboxypeptidase B) and some minor accompanying proteins, are eluted together in the sixth chromatographic fraction.

The separation of the two forms of pro-(carboxypeptidase A) is achieved in the second chromatography at pH 5.7, as shown in Fig. 2. On raising the NaCl gradient from 0.1 M to 0.35 M this procedure elutes sequentially monomeric pro-(carboxypeptidase A) (M_r approx. 45 000), the binary complex of pro-(carboxypeptidase A) and pro-(proteinase E) (M_r 45 000 + 27 000), impure pro-(carboxypeptidase B) (M_r approx. 47 000) and chymotrypsinogen (M_r approx. 30 000) (probably the C form; Gratecos *et al.*, 1969). The overlapping of the first two chromatographic peaks is usually slight, and it depends on how old the DEAE-Sephacrose used is and on the exact pH of the eluting buffer. Thus a slight increase in the pH of the eluting buffer, e.g. from 5.7 to 5.8, usually ruins the good separation between both pro-(carboxypeptidase A) forms. The great purity of these isolated pro-(carboxypeptidases A) and their identity with those previously isolated and character-

Fig. 2. Separation of the monomeric form and the binary complex form of pro-(carboxypeptidase A) by chromatography on DEAE-Sephacrose at pH 5.7

The column was loaded with fraction f from the previous chromatography at pH 8.0, containing both forms of pro-(carboxypeptidase A) and other accompanying proteins. The chromatography was developed with a linear gradient of 0–0.4 M-NaCl in the equilibration buffer, 40 mM-Tris/acetate, pH 5.7. Activities were measured as indicated in Fig. 1 legend, and the same symbols were used. The inset shows the electrophoretic analysis on sodium dodecyl sulphate/polyacrylamide gels at different stages of the isolation procedure: 1, pancreatic aqueous extract; 2, $(\text{NH}_4)_2\text{SO}_4$ precipitate of the aqueous extract; f, fraction f from Fig. 1; a'–d', sequential fractions from the chromatography shown in this Fig. 2; 0, mixture of markers (bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme).

ized by our group (Martínez *et al.*, 1981) were confirmed by electrophoresis (Fig. 2), *N*-terminal (which is lysine for both proteins) and amino acid analyses.

The final yield of pro-(carboxypeptidases A) isolated by the above method, starting from 100 g of defatted acetone-dried powder from pig pancreas, is 150–300 mg of monomeric form and 300–400 mg of the binary complex form, as estimated by absorbance measurements at 280 nm (Martínez *et al.*, 1983). These are average values calculated from 15 different preparations. The intrinsic peptidase activity on the artificial substrate benzoylglycyl-L-phenylalanine is 2.5 and 1.3 specific-activity units [μmol of transformed substrate/min per mg of pro-(carboxypeptidase A)] respectively. The peptidase activity of both forms rises to about 35 specific-activity units after complete activation with trypsin, a value that agrees closely with that reported for isolated carboxypeptidase A (Folk & Schirmer, 1963). It may be noted that the amount of pro-(carboxypeptidase A) obtained in each particular preparation was much more variable than the amount of pro-(carboxypeptidase A) binary complex obtained. This could be related to breed differences between the pigs processed in the slaughterhouse and/or to their diet. No systematic experiments have been carried out to test these hypotheses, the second of which has been proved to be true for other secretory proteins (Desnuelle *et al.*, 1962; Wicker *et al.*, 1984).

The main improvements of our method, compared with others previously reported for the preparation of pig pancreatic pro-(carboxypeptidases A), lie in the elimination of erratic degradations in these proteins during their isolation, in the substantial shortening of the preparative procedure, and in the higher resolution and purity of the isolated fractions. Thus the method of Folk & Schirmer (1963, 1965) requires more separation steps, requires double the analysis time (8–10 days instead of 4 days) and does not resolve the two forms of the proenzyme. Furthermore, the method of Kobayashi *et al.* (1978, 1981) is also much longer, uses a dangerous inhibitor of proteolysis, and does not resolve pro-(carboxypeptidase A) and pro-(carboxypeptidase B) well.

Isolation of carboxypeptidases A

Electrophoretic analyses on polyacrylamide gels in the presence and in the absence of sodium dodecyl sulphate indicate that the carboxypeptidase A fraction obtained from the first chromatography at pH 8.0 (Fig. 1, fraction e) is heterogeneous. The appearance of three main bands after electrophoresis in the absence of sodium dodecyl sulphate (Fig. 3 inset, lane 1)

suggests that this fraction contains a mixture of carboxypeptidases A₁, A₂ and A₃, proteins reported by Folk & Schirmer (1963) and probably generated by autolysis in the preparative procedure. To isolate carboxypeptidases A₁ and A₂ in a pure and homogeneous form, we found it more convenient and easier to reproduce if we used pro-(carboxypeptidase A) as a starting material (see below) rather than to separate them by chromatography from the above mixture, as done by Folk & Schirmer (1963). No attempts were made to isolate carboxypeptidase A₃, for which a preparative method has been reported by Koide *et al.* (1981).

To isolate carboxypeptidases A₁ and A₂, the binary complex of pro-(carboxypeptidase A) and pro-(proteinase E) was subjected to a limited proteolysis with trypsin. A subsequent chromatography on DEAE-Sephacel at pH 5.8, in the presence of 7 M-urea, allows the sequential separa-

Fig. 3. Chromatographic separation on DEAE-Sephacel at pH 5.8, in the presence of 7 M-urea, of carboxypeptidases A₁ and A₂ generated from a tryptic digest of pro-(carboxypeptidase A) binary complex with pro-(proteinase E). After the addition of soya-bean trypsin inhibitor, the tryptic digest of the zymogen was loaded on to the column. Carboxypeptidase A₁ (fraction a'') and carboxypeptidase A₂ (fraction b'') and accompanying proteins were eluted by a two-step salt gradient of 0–0.07 M-NaCl and 0.07–1 M-NaCl (.....) in 40 mM-sodium phosphate buffer, pH 5.8, containing 7 M-urea. Proteinase E was eluted when the column was washed with 2 vol. of initial equilibration buffer before the salt gradient was started. Abbreviation: STI, soya-bean trypsin inhibitor. The inset shows the polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate of: 1, the mixture of carboxypeptidases A found in fraction e of Fig. 1; 2 and 3, fractions a'' and b'' of this Fig. 3; 4, carboxypeptidases A generated from pro-(carboxypeptidase A) by tryptic digestion (15 min, 20:1 proenzyme/trypsin molar ratio) carried out before the chromatography shown.

tion of active proteinase E, carboxypeptidase A₁, carboxypeptidase A₂, activation peptide and soya-bean trypsin inhibitor (see Fig. 3 and the Materials and methods section for details). The presence of urea in the medium prevents the strong binding of the generated activation peptide to carboxypeptidase A (San Segundo *et al.*, 1982) and the co-elution of both proteins. When urea is removed by dialysis, both carboxypeptidases A recover their benzoylglycyl-L-phenylalanine-splitting activity, which reaches the value of 37 $\mu\text{mol/min}$ per mg reported by Folk & Schirmer (1963) for the specific activity of these proteins. With this preparative procedure, about 20 mg of carboxypeptidase A₁ and 23 mg of carboxypeptidase A₂ are obtained from 100 mg of pro-(carboxypeptidase A) binary complex. These carboxypeptidases A were found respectively to possess alanine and threonine as *N*-terminal residue, and hence our previous assignment was confirmed as being the A₁ and A₂ forms of pig carboxypeptidase A reported by Folk & Schirmer (1963), equivalent to homologous bovine α - and β -forms (Petra, 1970; Koide *et al.*, 1981).

The above separation method is used at present in our laboratory in the isolation of the primary and secondary activation peptides of pro-(carboxypeptidases A) generated at different times in the drastic proteolytic activation of these proenzymes. The characterization of the sequence, conformation and functional properties of those carboxypeptidases A and activation peptides should clarify the molecular events in the activation process of monomeric pro-(carboxypeptidase A), which is poorly understood compared with that of other pro-proteinases (e.g. serine pro-proteinases) (Neurath, 1984). The effect of the formation of complexes of zymogens of endoproteinases with pro-(carboxypeptidase A) on the activation process of the latter can also be studied, at the molecular level, more easily in the pig system than in other systems in which only oligomeric forms of pro-(carboxypeptidase A) are present (e.g. bovine) [see San Segundo *et al.* (1982) and Vendrell *et al.* (1982) for discussion]. The preparative procedure described above also allows the isolation of a highly pure and active proteinase E once urea is removed, and is therefore an alternative prepara-

tive method to that of Kobayashi *et al.* (1981). Carboxypeptidases A₁ and A₂ are also obtained if monomeric pro-(carboxypeptidase A) is used instead of the binary complex with pro-(proteinase E) as starting material.

We thank Dr. Blanca San Segundo for her collaboration in the first steps of this work. We are also indebted to the Fundación M. F. Roviralta for kind help. This work has been supported by Grant 0338/81 from the CAICYT, Ministerio de Educación y Ciencia, Spain.

References

- Creighton, T. E. (1979) *J. Mol. Biol.* **129**, 235–264
- Desnuelle, P., Reboud, J. P. & Abdeljlil, A. B. (1962) *Ciba Found. Symp. Exocrine Pancreas Norm. Abnorm. Functions*
- Folk, J. E. & Schirmer, E. W. (1963) *J. Biol. Chem.* **238**, 3884–3894
- Folk, J. E. & Schirmer, E. W. (1965) *J. Biol. Chem.* **240**, 181–192
- Gratecos, D., Guy, O., Rovey, M. & Desnuelle, P. (1969) *Biochim. Biophys. Acta* **175**, 82–96
- Kobayashi, R., Kobayashi, Y. & Hirs, C. H. W. (1978) *J. Biol. Chem.* **253**, 5526–5530
- Kobayashi, R., Kobayashi, Y. & Hirs, C. H. W. (1981) *J. Biol. Chem.* **256**, 2460–2465
- Koide, A., Yoshizana, M. & Kurachi, K. (1981) *Eur. J. Biochem.* **117**, 383–388
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Martínez, M. C., Avilés, F. X., San Segundo, B. & Cuchillo, C. M. (1981) *Biochem. J.* **197**, 141–147
- Martínez, M. C., Nieuwenhuysen, P., Clauwaert, J. & Cuchillo, C. M. (1983) *Biochem. J.* **215**, 23–27
- Moore, S. & Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831
- Neurath, H. (1984) *Science* **224**, 350–357
- Petra, P. H. (1970) *Methods Enzymol.* **19**, 460–508
- San Segundo, B., Martínez, M. C., Vilanova, M., Cuchillo, C. M. & Avilés, F. X. (1982) *Biochim. Biophys. Acta* **707**, 74–80
- Scheele, G. A. (1975) *J. Biol. Chem.* **250**, 5375–5385
- Schwert, G. W. & Takenaka, Y. (1955) *Biochim. Biophys. Acta* **16**, 576–583
- Vendrell, J., Avilés, F. X., San Segundo, B. & Cuchillo, C. M. (1982) *Biochem. J.* **205**, 449–452
- Wicker, C., Puigserver, A. & Scheele, G. (1984) *Eur. J. Biochem.* **139**, 381–387