

## A Bovine Pancreatic Enzyme Catalyzing the Conversion of Proinsulin to Insulin

(amino acid analysis/gel electrophoresis/tryptic activity/molecular weight)

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**ABSTRACT** An enzyme that catalyzes the conversion of bovine proinsulin to insulin has been purified from a bovine pancreatic extract. The product of conversion was identified as insulin by amino acid analysis and determination of carboxyl terminal amino acid residues. The purified enzyme preparation showed one major protein band on polyacrylamide gel disc electrophoresis; it had a molecular weight of about 70,000 and an isoelectric point (pI) at a pH of 4.82.

The biosynthesis of insulin involves the initial synthesis of a single-chain polypeptide that is its precursor, proinsulin (1-6). The primary structure of proinsulin (7, 8) suggests that insulin may be formed by the removal of the connecting peptide from proinsulin. This consideration is supported by the isolation of at least two double-chain intermediate forms of proinsulin (9), and by the observation that intact C-peptide (connecting peptide minus the two pairs of basic amino acids) is evidently secreted into the circulation (10). The conversion process appears to take place in the  $\beta$ -granule fraction of the islets (11, 12), although the nature of the enzyme(s) so involved is not known. Theoretically, insulin may be formed from an initial tryptic cleavage of proinsulin followed by the removal of the B<sub>31</sub> arginine residue by a carboxypeptidase. The present communication reports on the isolation and purification of an enzyme from bovine pancreatic extract that catalyzes the conversion of bovine proinsulin to intact insulin. A preliminary report of this study has appeared (13).

### MATERIALS AND METHODS

#### Purification of enzyme

The starting material was an ammonium sulfate precipitate which did not contain insulin activity and was discarded from the production and purification of bovine insulin by the Connaught Medical Research Laboratories. The precipitate was extracted at 4°C with water at pH 8.0, and the pH of the extract was lowered to pH 3.1, when a precipitate formed. The supernatant fraction was then further treated with ammonium sulfate at 20% saturation. The resulting precipitate was dissolved in water and dialyzed. The dialyzed material was acidified to about pH 3 and chromatographed through Sephadex G-50 equilibrated to 1 M acetic acid. The fractions that were eluted before bovine proinsulin were pooled and precipitated at 50% saturation of ammonium

sulfate and dialyzed. The dialyzed material was clarified by acidification and lyophilized.

The lyophilized powder was further purified by gel filtration through Sephadex G-100 in 1 M acetic acid. The fractions with enzyme activity were pooled and further purified by application to a column for ion-exchange chromatography on DEAE-cellulose in 0.01 M Tris·HCl buffer, pH 8.0 and were eluted with a gradient of NaCl in the same buffer. The active fractions were pooled and further purified by gel filtration through Sephadex G-50 equilibrated to 0.01 M Tris·HCl, pH 8.0.

#### Assays of enzyme activities

*Hydrolysis of BzArgAnilNO<sub>2</sub>*. The reaction mixture contained 0.9 ml of 0.4 M Tris·HCl buffer (pH 8.0) with 0.05 M

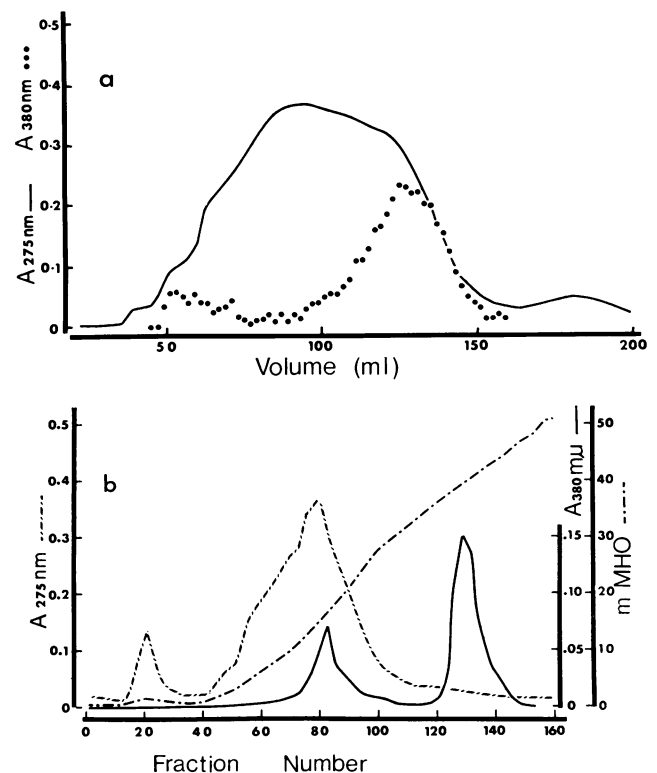


FIG. 1. (a) Gel filtration of crude enzyme preparation through a column (2.5 cm × 40 cm) of Sephadex G-100 equilibrated to 1 M acetic acid. (b) Chromatography of the pooled fractions from 1a on a DEAE-cellulose column (1.5 cm × 40 cm).

Abbreviations: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; BzArgAnilNO<sub>2</sub>,  $\alpha$ -N-benzoyl-DL-arginine-p-nitroanilide·HCl.

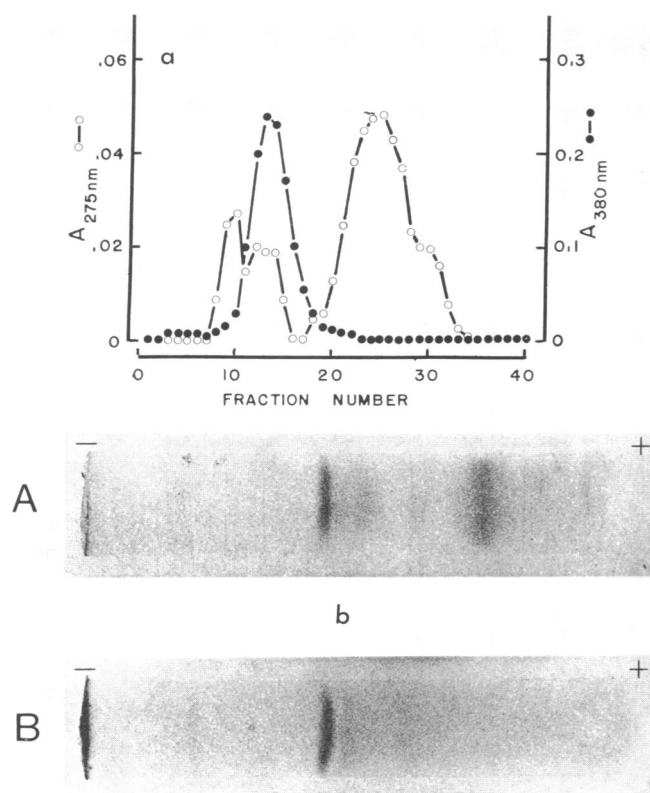


FIG. 2. (a) Gel filtration of the enzyme preparation after DEAE-cellulose purification through a column (1.5 cm  $\times$  40 cm) of Sephadex G-50 equilibrated to 0.01 M Tris buffer, pH 8.0; 1.0 ml/fraction. (b) Polyacrylamide disc gel (10% gel, pH 8.9) electrophoresis of the enzyme preparation after DEAE-cellulose chromatography (A) and after further purification through Sephadex G-50 (B).

CaCl<sub>2</sub>, 0.1 ml of 1 mM BzArgAnilNO<sub>2</sub>, and aliquots of the fractions to be assayed. The mixture was incubated at 37°C for 2 hr and an increase in the absorbance at 380 nm was measured.

**Conversion of Proinsulin to Insulin.** The reaction mixture contained 50  $\mu$ l of 0.2 M Tris·HCl buffer (pH 8.0), 50  $\mu$ g of proinsulin, aliquots of fractions to be assayed, and water, in a final volume of 250  $\mu$ l. The single-chain bovine proinsulin was purified by ion-exchange chromatography to a state that gives a single band on urea-polyacrylamide gel electrophoresis (14). Incubation was in small, siliconized glass test tubes at 37°C for 2 hr. After incubation, 40- $\mu$ l aliquots were analyzed by urea-polyacrylamide gel electrophoresis at pH 9.2 (5).

**Carboxypeptidase B Activity** was assayed according to Folk *et al.* (15).

#### Aminoacid analysis and carboxyl-terminal determination

Aminoacid analyses were performed on a Beckman-Spinco Amino Acid Analyzer, model 120-C. Samples were hydrolyzed with 6 N HCl at 110°C for 18–22 hr.

Carboxyl-terminal determination was done with carboxypeptidase A or B. The incubation period was 4 hr at 37°C. Carboxypeptidase A was treated before use according to Ambler (16). The terminal aminoacid residues released were directly identified on the aminoacid analyzer.

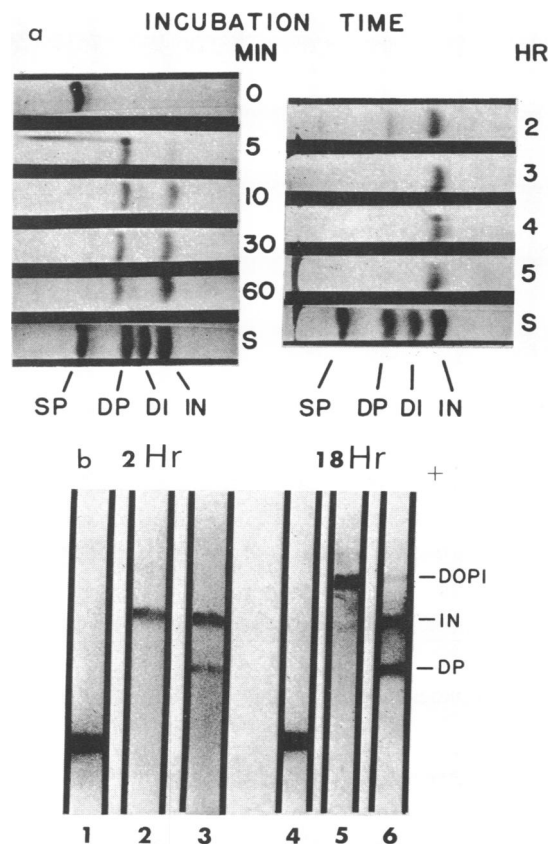


FIG. 3. (a) Conversion of bovine proinsulin to insulin by the purified enzyme preparation, as demonstrated by urea-disc gel electrophoresis at pH 9.2. Single-chain proinsulin (SP), double-chain intermediate (DP), dimer of insulin (DI), and insulin (IN). S: electrophoresis of standards. FIG. 3. (b) Effect of Zn<sup>++</sup> on the enzymic conversion of bovine proinsulin to insulin. 1 and 4, proinsulin substrate; 2 and 5, incubation without Zn<sup>++</sup>; 3 and 6, incubation with Zn<sup>++</sup>. DP, IN, and DOPI indicate the corresponding band positions upon disc electrophoresis of double-chain proinsulin intermediate, insulin, and desoctapeptide insulin, respectively.

#### Molecular weight determination

The method of Weber and Osborn (17), with sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used. Bovine serum albumin, crystalline pepsin, and crystalline trypsin were used as reference standards.

## RESULTS

#### Purification and properties of the enzyme

During purification, fractions were assayed for their enzymic activity for the hydrolysis of BzArgAnilNO<sub>2</sub> (a measurement of tryptic activity) and for the conversion of proinsulin to insulin.

Gel filtration of the proteins not retarded by Sephadex G-50 showed that the enzyme was retarded by Sephadex G-100 (Fig. 1a). DEAE-cellulose ion-exchange chromatography further resolved the material into two components with tryptic activity (Fig. 1b). However, only the component eluted at the higher salt concentration was able to convert proinsulin to insulin. This active material showed two major protein bands on polyacrylamide disc electrophoresis (Fig. 2b (A)). This material was further purified on Sephadex G-50 (Fig.

TABLE 1. *Aminoacid analysis of the product of enzymic conversion of proinsulin*

Product		Bovine insulin residues/mol (Theoretical)
Amino acid	Residues/mol*	
Lys	1.0 (1)	1
His	2.0 (2)	2
Arg	1.2 (1)	1
Asp	3.1 (3)	3
Thr	0.9 (1)	1
Ser	3.2 (3)	3
Glu	7.1 (7)	7
Pro	1.1 (1)	1
Gly	4.2 (4)	4
Ala	2.8 (3)	3
Cys/2	†	6
Val	4.6 (5)	5
Ile	0.5 (1)	1
Leu	6.3 (6)	6
Tyr	3.7 (4)	4
Phe	2.5 (3)	3

\* Figures in parentheses are nearest integer.

† Not determined.

2a); the purified enzyme preparation showed one major protein band on polyacrylamide gel electrophoresis (Fig. 2b (B)).

The isoelectric point of the purified enzyme was pH 4.82, as determined by isoelectrofocusing in 1% Ampholine that contained 6 M urea. The enzyme was active at pH values between 6.5 and 8.5, and was inactivated by boiling for 10 min. The enzyme was inhibited by the following inhibitors: Kunitz pancreatic, Greene's porcine pancreatic, soybean, and lima bean, but not by TPCK. Diisopropylfluorophosphate was also inhibitory. The molecular weight of the enzyme was estimated to be about 70,000 by gel electrophoresis. The enzyme demonstrated no carboxypeptidase B activity.

#### Identification of the enzymic product

As shown in Fig. 3a, the single-chain bovine proinsulin was rapidly and completely converted (within 5 min) to a major component having the same electrophoretic mobility as a double-chain proinsulin intermediate. This component was then converted slowly to a product with the same electrophoretic mobility as bovine insulin. Since the techniques of electrophoresis and chromatography cannot distinguish between intact insulin and des-alanyl insulin, a product of tryptic hydrolysis of proinsulin, the product of the enzymic reaction was isolated and purified by gel filtration on Sephadex G-50 followed by ion-exchange chromatography on DEAE-Sephadex in 7 M urea for further identification. The homogeneity of the product was established by high-voltage paper electrophoresis on Whatman 3MM paper in 30% formic acid (2 kV, 2 hr). The product was as active as insulin in lowering the blood sugar of rabbits.

Aminoacid analysis of the purified product established its identity as intact bovine insulin (Table 1). Carboxyl-terminal determination with carboxypeptidase A showed the presence of asparagine and alanine (Table 2). Lysine was not detected with carboxypeptidase B.

Aminoacid analysis of the incubation mixture during the course of conversion of proinsulin to insulin by the purified

TABLE 2. *Hydrolysis of conversion product with carboxypeptidase A*

	Amino acids released ( $\mu$ mol)	
	Asparagine	Alanine
Enzyme blank	0	0
Product	Trace	0.019*

\* Total protein analyzed was about 0.036  $\mu$ mol. Protein/enzyme was about 100/1 by weight.

enzyme showed only the appearance of arginine; no other amino acids were detected.

#### Effect of zinc on the conversion of proinsulin to insulin

The conversion of single-chain bovine proinsulin to insulin was greatly influenced by zinc. The addition of zinc, in the ratio of one atom of zinc to one molecule of proinsulin, inhibited the conversion of the intermediate to insulin; Zn had no effect on the formation of the intermediate from the precursor. Incubation of the precursor in the absence of added zinc for 18 hr with the purified enzyme resulted in the degradation of insulin to des-octapeptide insulin, whereas the addition of zinc prevented to a great extent such degradation (Fig. 3b).  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$ , and  $\text{Mn}^{++}$  had no effect on the enzymic conversion. The hydrolysis of  $\text{BzArgAnilNO}_2$  by this enzyme, however, was not inhibited by zinc.

#### DISCUSSION AND CONCLUSION

I have shown that an enzyme obtained from a by-product fraction of insulin production can catalyze the conversion of bovine single-chain proinsulin to insulin. The identification of the conversion product as intact insulin is based on: its electrophoretic mobility in urea-polyacrylamide gel; its aminoacid composition; its carboxyl terminals being asparagine and alanine; and its hypoglycemic effect in rabbits.

The enzymic conversion appears to occur in a two-step reaction: a fast hydrolysis to an intermediate followed by a slow cleavage of the intermediate to insulin. The connecting peptide apparently is released intact, since only arginine, and no other amino acids, was detected during hydrolysis. The purity of the final enzyme preparation suggests that the entire process might be catalyzed by a single protein. This is further supported by the absence of carboxypeptidase B activity in the enzyme preparation. The effects of inhibitors on the activity of this enzyme indicate its tryptic characteristics. However, its pI and molecular size clearly distinguish it from pancreatic trypsin or trypsinogen.

The effect of zinc on the conversion process may be due to the interaction of zinc with proinsulin, since zinc did not affect the hydrolysis of  $\text{BzArgAnilNO}_2$  by this enzyme. Since interaction with zinc would change the conformation of the precursor, the intermediate, and the product insulin, the  $\text{B}_{22}$  arginine bond may have been protected from hydrolysis that would have formed desoctapeptide insulin. This consideration is in accord with the observation of Wang and Carpenter (18) on the hydrolysis of zinc-free insulin by trypsin. Frank and Veros (19) also speculated on the role of procine proinsulin-zinc complex in the conversion of proinsulin to insulin.

Although the enzyme preparation is of pancreatic origin, it remains to be established whether it plays a role in the *in vivo* conversion of proinsulin to insulin in the islet  $\beta$ -cells.

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