

Isoelectric Focusing of Insulin and Insulin Derivatives

BENNY SABROE WELINDER

Steno Memorial Hospital, Research Laboratory, Gentofte, Denmark

By isoelectric focusing in 6 M urea insulin was found to contain a main fraction and three to four minor fractions. The isoelectric point (pI) for the main fraction was found to be 6.15. pI for the main fraction of insulin iodinated to 1 and 3 $^{125,127}\text{I/mol}$ were found to be 6.12 and 6.07, respectively. By isoelectric focusing of insulin iodinated to approximately 0.7 $^{125}\text{I/mol}$ with high specific radioactivity in solutions without urea pI for the main fraction was found to be 5.74.

Isoelectric focusing of carbamylated and deamidated insulins is reported. The reason for the difference in pI values measured by isoelectric focusing and by other techniques, as well as the identity of the minor fractions, are discussed.

It has been demonstrated by several methods that crystalline insulin contains some minor protein components. This was shown by countercurrent distribution,¹ partition column chromatography,² electrophoresis,³ ion exchange chromatography,⁴ and disc-electrophoresis.⁵

Recently insulin was investigated by isoelectric focusing in polyacrylamide gels. Lewin⁶ found that all the insulin preparations examined gave more than one fraction, except a ten times crystallized bovine insulin preparation. Percival *et al.*⁷ found one main and five minor fractions using the same technique.

The main purpose of this study was to investigate insulin and iodinated insulin by isoelectric focusing in a density gradient column as described by Vesterberg and Svensson⁸ and to identify the different components. Since it is known that deamidated insulin normally forms the major part of the minor components found in insulin¹ and since insulin in urea solution may be carbamylated spontaneously,⁹ special attention was paid to these insulin derivatives.

MATERIALS AND METHODS

Insulin. Crystalline pig insulin, Nordisk Insulinlaboratorium, batch 1921 A, containing approximately 7% H_2O stored at 4°C was used for all the preparations. The zinc content was 0.53% of dry weight; the biological activity, determined by the mouse convulsion test, was 25 IU/mg.

Iodinated insulin. Iodinated insulin with an iodine content of 1 and 3 $^{125,127}\text{I}$ /mol insulin and insulin, iodinated to approximately 0.7^{125}I /mol insulin with high specific radioactivity (approximately 100 mC/mg) were prepared according to Brunfeldt *et al.*¹⁰

Carbamylated insulin. 100 mg insulin was dissolved in 10 ml 8 M urea. The solution was incubated for 24 h at 37°C. The mixture was applied directly to the isoelectric focusing column.

Partially deamidated insulin. 100 mg insulin was dissolved in 10 ml 0.1 N HCl and incubated at 37°C for 72 h as described by Sundby.¹¹ The mixture was gel filtrated on Sephadex G-25 fine (100 × 3.2 cm i.d.). The protein fraction was collected and freeze-dried.

Isoelectric focusing. Isoelectric focusing was performed as described by Vesterberg and Svensson⁸ except for the addition of urea to all solutions to a final concentration of 6 M in almost all the experiments described here to prevent precipitation of the insulin. LKB 8100-10 and LKB 7900-2 columns were used. In all experiments 1% Ampholine carrier ampholytes were used. A voltage of 300 V was applied for a period of 3-6 h followed by a voltage of 1000 V for 72 to 120 h. The columns were cooled by tap water (approximately 16°C).

The columns were emptied at an elution rate of 60 ml/h and the eluate was collected in fractions of 2.5 ml. The absorption at 280 nm was measured either continuously during the emptying (LKB Uvicord II) or in each fraction (Beckmann DU spectrophotometer). Radioactivity was counted in a Philips scintillation counter (well type). pH in each fraction was measured with a Radiometer 26 pH-meter. All measurements outside the column were made at room temperature, about 22°C. Blank experiments (without protein) have been carried out to estimate the self-absorption of the ampholytes and the sucrose.¹²

Disc-electrophoresis. Disc-electrophoresis was performed according to Davis¹³ with one modification: pH in the running gel solution was adjusted to 9.15 instead of adjusting the running gel buffer to pH 8.90. This was found to give a better separation of the anodal fractions. The amount of protein used was 0.6 mg and the gels were stained with Coomassie Brilliant Blue.

RESULTS

Insulin focused in a pH 4-6 gradient showed four fractions (Fig. 1), one main fraction (III) and three minor fractions, two anodal (I and II) and one cathodal (IV) to the main fraction. The pI values for the fractions are given in Table 1. When a pool of fractions II, III, and IV was rerun, a small amount of fraction I was found again. When insulin was focused in a pH 5-7 gradient, two cathodal fractions with pI 6.60 and 6.71 were found.

Disc-electrophoresis of an aliquot of the main fraction showed that it contained the major component of insulin and proinsulin. Disc-electrophoresis of the cathodal fraction IV showed that it was separated in two fractions, both having approximately the same position as "intermediate insulin".¹⁴

Almost identical results were obtained by isoelectric focusing of iodinated insulin derivatives. Insulin iodinated to 1 and 3 $^{125,127}\text{I}$ /mol insulin both showed one main fraction (III), two anodal fractions (I and II) and one cathodal fraction (IV) when they were focused in a pH 4-6 gradient as well as in a pH 5-7 gradient (Fig. 2). The pI values are given in Table 1.

Since it may be expected that insulin in high dilution is soluble even at pI without addition of urea, a few experiments were made using 100 ng insulin iodinated to approximately 0.7^{125}I /mol with a specific radioactivity of approximately 100 mC/mg. When isoelectric focusing was performed in solutions without urea, pI for the main fraction was found to be 5.71 and 5.76 in two experiments. When the focusing was made in 6 M urea, pI for the main

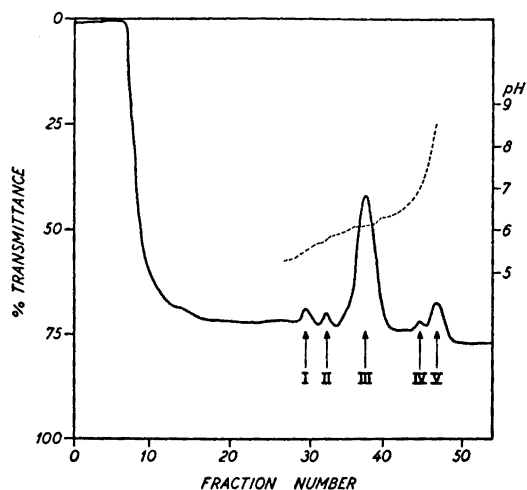


Fig. 1. Isoelectric focusing in 6 M urea of 25 mg pig insulin. The anode is to the left. --- pH; ——— E^{280} . The peak called V is an artefact arising from the electrode solution.

fraction was found to be 6.15. A large amount of radioactivity was found in the electrode solutions, either due to reaction with these or due to deiodination during the experiment.

The pH gradient formed in experiments using 6 M urea was different from the one formed in experiments without urea. It seems that the addition of urea causes a parallel displacement of the pH gradient of approximately 0.5 pH unit towards higher values.

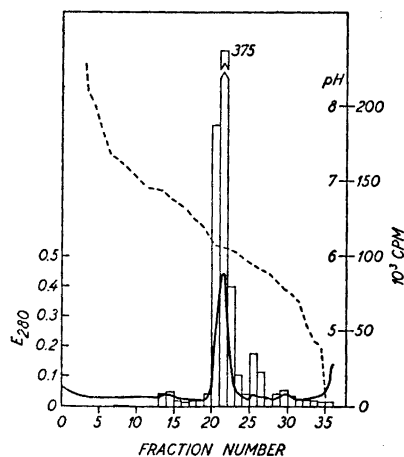


Fig. 2. Isoelectric focusing in 6 M urea of 50 mg insulin, iodinated to 1 $^{125,127}\text{I}$ /mol. The anode is to the right. --- pH; ——— E^{280} . The histogram shows the distribution of radioactivity.

Isoelectric focusing of carbamylated insulin showed two main fractions (I and II) and minor one (III), the minor one having the same pI value as fraction III from unmodified insulin (Fig. 3). The two main fractions probably correspond to insulin that has been carbamylated in one and two amino groups, the latter being the most anodal.

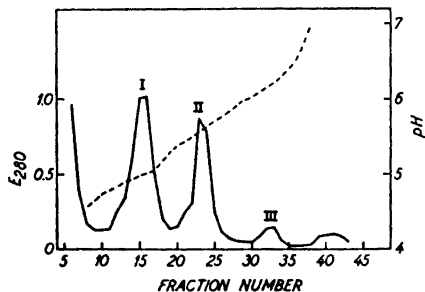


Fig. 3. Isoelectric focusing in 6 M urea of 25 mg carbamylated insulin. The anode is to the left. --- pH; ——— E^{280} . The peak outside the pH gradient is an artefact arising from the electrode solution.

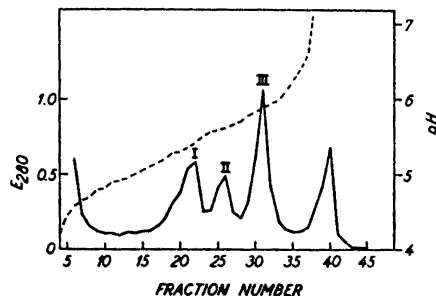


Fig. 4. Isoelectric focusing in 6 M urea of 25 mg deamidated insulin. The anode is to the left. --- pH; ——— E^{280} . The peak outside the pH gradient is an artefact arising from the electrode solution.

Partially deamidated insulin showed one main fraction (III) and two minor fractions, (I and II) (Fig. 4). The main fraction corresponds approximately in pI value to fraction III from unmodified insulin, the two smaller fractions are probably insulin that has lost one and two amide groups, the latter being the most anodal.

Table 1. pI values for insulin and insulin derivatives. The numbers of the fractions correspond to the numbers shown on the figures. S. D. for the measurements of the main fraction (fraction III) for insulin and insulin iodinated to 1 and 3 $^{125,127}\text{I}$ /mol are 0.04 ($n=8$), 0.05 ($n=11$), and 0.04 ($n=10$) pH units, respectively.

	I	II	III	IV
Insulin	5.56	5.82	6.15	6.74
1 I/mol	5.53	5.85	6.12	6.73
3 I/mol	5.56	5.86	6.07	6.60
Carbamylated insulin	5.00	5.56	6.14	
Deamidated insulin	5.62	5.82	6.09	

Disc-electrophoresis showed that when insulin is carbamylated, it contains a fraction with the same electrophoretic mobility as the presumed desdiamidinsulin.

CONCLUSIONS

Estimated from the pI values, fraction I of unmodified insulin most likely contains monocarbamylated insulin and presumed desdiamidinsulin. This is supported by disc-electrophoresis, where these two fractions showed the same electrophoretic pattern.

Fraction II of unmodified insulin most likely contains desmonoamidinsulin. Disc-electrophoresis of insulin showed that it contains desmonoamidinsulin as well as presumed desdiamidinsulin.

Fraction III of unmodified insulin most likely contains the major component of insulin and proinsulin as shown by disc-electrophoresis.

Fraction IV of unmodified insulin contains two components, both having the same electrophoretic pattern as "intermediate insulin".

It has been shown that isoelectric focusing of unmodified insulin for 96–120 h at 16°C is followed by carbamylation since an amount of material with the same pI value as fraction I was found when a pool of fractions II, III, and IV was rerun.

From the experiments with iodinated insulin derivatives the conclusions to be drawn are probably analogous with those mentioned for unmodified insulin.

DISCUSSION

The pI value for insulin in 6 M urea at 22°C was found to be 6.15, S.D. 0.04, $n = 8$ by isoelectric focusing. This value refers to zinc free insulin since insulin was freed from zinc because of the urea and Ampholine ampholytes in the solutions. The pI value does not agree with earlier estimations using other methods.

Wintersteiner and Abrahamson¹⁵ found by electrophoresis pI for insulin to be 5.3–5.35. Howitt and Prideaux¹⁶ found by moving boundary electrophoresis pI to be 5.4. Tanford and Epstein¹⁷ found by titration pI to be 5.60 for zinc free as well as for zinc insulin. By nephelometric experiments Ziegler and Lipmann¹⁸ found pI to be 5.39 ± 0.06 . All the measurements were carried out at room temperature.

The reason for the difference between these values and the value found by isoelectric focusing may be explained by the assumption that (1) urea influences the pI of insulin; (2) the earlier methods were carried out in solutions with a considerable ion strength whereas isoelectric focusing refers to an ion strength of approximately zero.

Concerning (1), Gerber and Barandum¹⁹ focused soluble Bence Jones proteins and found no changes in pI values when they changed from 0 to 5 M urea, but they do not refer to any experiments in 6 M urea. Wadström²⁰, who focused deoxyribonuclease, and Harris *et al.*,²¹ who focused yeast aldolase, found the same number of peaks when they focused in 0 and 6 M urea; however, these authors do not mention whether they found any changes in pI values of these fractions.

It should be noticed that 6 M urea, having a pH of 8.37, influences the pH of a standard buffer solution. When Radiometer S 1001 concentrated buffer

was made up with water and 6 M urea, respectively, a pH of 6.50 and 6.79 was found.

Concerning (2), Svensson,²² who focused hemoglobin, found a pI value which was higher than that found by moving boundary electrophoresis. Also Vesterberg and Svensson,⁸ who focused myoglobin, found an increased pI value compared with that found by moving boundary electrophoresis. Both explained the higher pI values as a result of the lower ion strength in the isoelectric focusing experiments.

From the experiments described here it is clear that pI of insulin, iodinated to 0.7 ¹²⁵I/mol is influenced by urea. Since no significant pI difference was found between insulin and insulin iodinated to 1 ^{125,127}I/mol it may be concluded that pI of insulin also is influenced by urea. This fact explains most of the difference in pI values found by isoelectric focusing and by other methods.

It may therefore be concluded that the difference noticed between pI values measured by isoelectric focusing in 6 M urea and by other methods may be explained partly by the influence of the urea, partly by the difference in ion strength.

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