CHARACTERIZATION BY ISOELECTRIC FOCUSING OF PIG HEART ASPARTATE AMINOTRANSFERASE

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1. Introduction

In 1965 Martinez-Carrion et al. [1] showed that purified preparations of pig-heart aspartate aminotransferase contain multiple catalytically active subforms, indicated as α, β, γ, and δ, in the order of increasing anionic character. Later the same authors [2] described a simple chromatographic procedure which allows a partial separation of the four subforms. The proteins present in each active chromatographic fraction, although identical in molecular weight, amino acid composition and peptide map, differ in their specific activity [2]. Similar results were reported by Banks et al. [3], who suggested that the enzyme subforms may arise from a conformation change. As a matter of fact, the origin and nature of aspartate aminotransferase heterogeneity are still obscure; a progress towards their elucidation might be obtained through chemical and physico-chemical studies of homogeneous subforms.

This communication deals with some preliminary results on the fractionation and characterization of porcine aspartate aminotransferase subforms by isoelectric focusing [4, 5], a tool which has been very valuable in the study of a large number of heterogeneous proteins [6].

2. Experimental

Aspartate aminotransferase was prepared following the method B of Martinez-Carrion et al. [2]. The enzyme preparation had a specific activity of 850 units as assayed according to Banks et al. [3] ** and showed five enzymatically active bands when submitted to starch-gel electrophoresis [2]. The fifth band, moving ahead of δ and here called ϵ, has a very low enzymatic activity; it was constantly detected in the concentrated enzyme preparation used for the present investigation. The purified subform α, used for one electrofocusing experiment, was prepared by chromatography [2]; the sample was contained by a small amount of subform β. Before electrofocusing all enzyme samples were thoroughly dialysed against 1% glycine solution and then added with sucrose (250 mg/ml).

The isoelectric focusing experiments were performed with an LKB 8102 column, using as carrier ampholytes "Ampholine" purchased from the same firm. The column assembling and the preparation of the sucrose density gradient were made according to the LKB instruction sheet; unless otherwise stated the anode was positioned at the bottom. Carrier ampholytes of restricted pH range

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** Specific activities higher than those reported by Banks et al. [3] were consistently obtained throughout the purification procedure.

Table 1
Summary of the data for the characterization of porcine aspartate aminotransferase subforms by isoelectric focusing.

Exp. no.	pH range	Peaks	pH	Specific activity
		A	5.68	1,400
		B	5.54	900
1	5–7	C	5.43	800
		D	5.41	740
		E	5.35	650
		A	5.69	1,150
		B	5.54	1,020
2	5–7*	C	5.44	790
		D	5.40	680
		E	5.36	600
		A	5.68	1,100
		B	5.51	900
3	4–6	C	5.42	790
		D	5.38	680
		E	5.34	520
4	5–7	A	5.70	1,500
		B	5.52	1,400
		A	5.70	1,600
		B	5.52	1,370
5	5.3–6.1	C	5.42	830
		D	5.38	670
		E	5.22**	490

* In this experiment the polarity of the electrodes was inverted.
** The peak falls in a pH zone gradient not linear.

(5.3–6.1) were obtained by a preliminary 100 hr isoelectric focalization of a 4% solution of "Ampholine" covering the pH range 5–7.

The enzyme sample (15–20 mg/ml) was layered in the middle of the column by a syringe fitted with a teflon capillary tube. All electrofocusing experiments were performed for 120–140 hr at 2 ± 0.5°C by applying a maximum power of 6 W.

After each focalization run 2 ml fractions were collected from the bottom of the column; the enzyme activity and pH value of each fraction were determined. The pH was measured at 25°C with a radiometer pH-meter mod. TTT 1, equipped with the scale expander 630 T.

Electrofocusing patterns were obtained by plotting enzyme activity against elution volume; peaks were indicated as A, B, C and so forth, in the order of decreasing pH. Fractions belonging to the same peak were pooled, extensively dialysed against water and then against acetate buffer 0.05 M pH 5.4; finally their gel-electrophoretic behaviour [2] and specific activity were determined. It should be pointed out that in spite of the duration of the electrofocusing experiments, the recovery of the enzyme activity was always complete.

3. Results and discussion

Preliminary experiments of isoelectric focusing in the pH range 3–10 showed a broad tailed band of enzyme activity in the pH region around 5.6. Hence carrier ampholytes of restricted pH range were employed for further experiments, five of which are discussed below; the results are summarized in table 1.

As shown in fig. 1, five peaks were obtained when carrier ampholytes covering the pH range 5–7 were used. However, gel electrophoresis indicated that a certain amount of overlapping still occurred between the different subforms (insert fig. 1).

In order to exclude possible side effects caused by gravity and "Ampholine" composition, the polarity of the electrodes was inverted (Exp. 2), and carrier ampholytes covering the pH range 4–6 were used (Exp. 3). Five peaks were observed in both experiments and, within the limits of experimental error, pH and specific activity of each peak were identical to those found in Exp. 1. Furthermore, no significant change was observed in the gel-electrophoretic patterns.

When a purified sample of the subform was analysed in the pH range 5–7 (Exp. 4) only two sharp peaks, A and B, were observed, A being the most prominent. The gel-electrophoretic analysis showed that peaks A and B contain respectively the subforms α and β with a slight reciprocal contamination.

In order to increase the resolution of peaks A and B, a sample was run with carrier ampholytes covering the pH range 5.3–6.1 (Exp. 5). As shown in fig. 2, peaks A and B were completely resolved and quite homogeneous on gel electrophoresis; on the other hand, no improvement was observed in the resolution of peaks C, D and E.

By considering the gel-electrophoretic patterns and

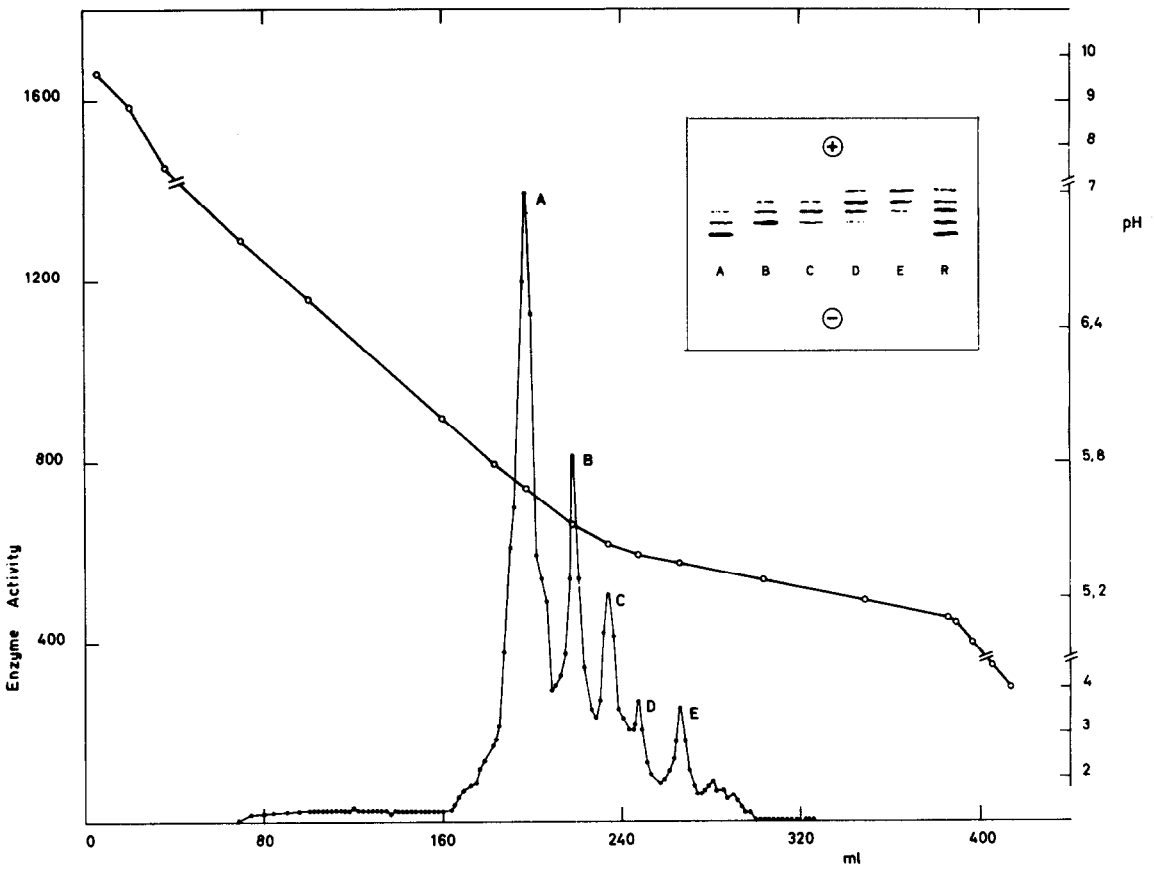

Fig. 1. Electrofocusing pattern of aspartate aminotransferase using carrier ampholytes pH 5–7 (Exp. 1). The insert shows the starch-gel electrophoresis of proteins corresponding to each focused peak (R = enzyme before electrofocusing).

and the specific activity of each peak, it is evident that a close correspondence exists between the focused peaks and the aspartate aminotransferase subforms. Therefore, on the assumption that the pH value of each peak corresponds to the isoelectric point, p*I*, of the focalized ampholyte [4, 5], it is possible to say that the porcine aspartate aminotransferase subforms are characterized by different p*I*'s and differ in their apparent net charge. A p*I* value of 5.69 ± 0.02 and of 5.53 ± 0.02 may be ascribed respectively to the subforms α and β; definite values of p*I* cannot be assigned to the subforms γ, δ and ϵ. The lack of resolution, even in the most restricted pH range used in this work indicates that these values must be very close to each other.

4. Conclusion

The electrofocusing technique in the present investigation has made it possible to determine the p*I* of the main aspartate aminotransferase subforms, for which no reliable values were hitherto available. In particular the high resolution focalization exhibits a distinct advantage over other described methods for preparing the subforms α and β; in fact, thanks to the procedure described in this paper, each subform was obtained completely free of traces of the other, and with a higher specific activity than that of the subforms α and β prepared in our Laboratory by the previously mentioned techniques [1–3]. Conditions for obtaining the subform γ, δ and ϵ as well,

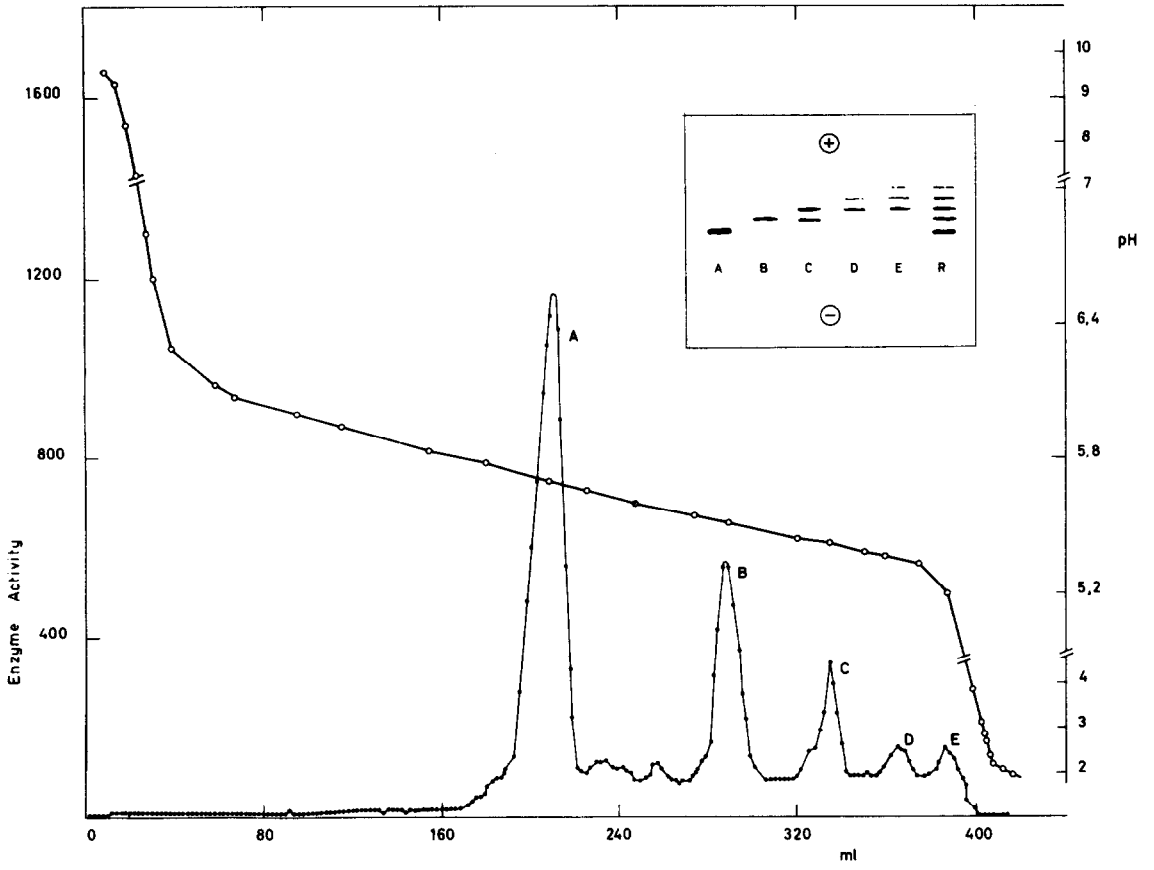

Fig. 2. Electrofocusing pattern of aspartate aminotransferase using carrier ampholytes pH 5.3–6.1 (Exp. 5). The insert shows the starch-gel electrophoresis of proteins corresponding to each focused peak (R = enzyme before electrofocusing).

in a state of high purity, are now under investigation.

The elctrofocusing technique is also being tested for the characterization of inactive aspartate aminotransferase subforms derived from the active subforms by aging [2] and by treatment with urea [3] or ammonium sulphate [7].

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