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Isolation of isoproteins from monoclonal antibodies and recombinant proteins by chromatofocusing

ALOIS JUNGBAUER*, CHRISTA TAUER, ELISABETH WENISCH, KAROLA UHL, JUTTA BRUNNER, MARTIN PURTSCHER, FRANZ STEINDL and ANDREA BUCHACHER

Institute of Applied Microbiology, University of Agriculture and Forestry and Biotechnology, Peter Jordanstrasse 82, A-1190 Vienna (Austria)

ABSTRACT

A fast protein liquid chromatographic method for the preparative separation of the various isoproteins is described. Highly purified human monoclonal antibodies, recombinant human superoxide dismutase and human superoxide dismutase from erythrocytes were used as starting material. The isoproteins were separated by chromatofocusing on Mono P columns. A very narrow pH gradient was applied to achieve complete separation of the isoproteins. The prepurification steps and the pretreatment of the samples to achieve optimum resolution are described in detail. The method is also applicable to extremely basic monoclonal antibodies (pI = 9). The successful separation was checked by isoelectric focusing in immobilized pH gradients (Immobilines). The future of these methods is discussed, because for many different biochemical and biophysical investigations pure and homogeneous isoproteins are necessary.

INTRODUCTION

Monoclonal antibodies[1–3] and many enzymes[4] exhibit strong microheterogeneity. Up to ten different isoproteins are observed by isoelectric focusing (IEF) in a monoclonal antibody sample. To investigate the microheterogeneity and the reasons for this biological phenomenon, the proteins must be separated into the various isoproteins. Owing to the separating power required, only a few methods are applicable for this separation problem. The most popular method is preparative isoelectric focusing[1]. The isoproteins are easily resolved. After staining part of the gel, the proteins must be extracted from the gel and often the yield of the extraction process is very low. Ion-exchange chromatography is also applicable to this separation problem, but its resolving power is inadequate for the complete separation of isoproteins with similar isoelectric points.

Chromatofocusing, first described by Slyterman and co-workers[5,6], separates proteins in order of their isoelectric points (pI). The column is filled with a weak ion exchanger. On tritrating the ion-exchange matrix with an amphoteric buffer, a linear

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pH gradient is formed in the column. Focusing takes place and results in peak sharpening. The samples are eluted in high concentration and are highly resolved.

A human monoclonal antibody exhibiting as very high p*I* and a native and recombinant superoxide dismutase (SOD) exhibiting a low p*I* were used as model substances. All three model substances displayed a characteristic microheterogeneity pattern in IEF. The human monoclonal antibody and the SOD were highly purified. This material was used for chromatofocusing on Mono P columns. The successful separation was demonstrated by IEF.

EXPERIMENTAL

Starting material

As starting material human monoclonal antibodies were prepared as described by Jungbauer *et al.*[7]; human recombinant SOD and human SOD from erythrocytes, as described by Weselake *et al.*[8], were used. The proteins were highly purified, with at least 99% purity. The samples were lyophilized from volatile buffers (10 m*M* ammonium formate, pH 8.0).

Chromatofocusing

Mono P chromatofocusing columns (Mono HR 5/20 and HR 10/30) from Pharmacia (Uppsala, Sweden) were used. The columns were connected to a fast protein liquid chromatographic (FPLC) system and chromatography was performed at flow-rates of 0.1 ml/min (HR 5/20) and 1.0 ml/min (HR 10/30).

For chromatofocusing of the human monoclonal antibody, 25 m*M* diethylenamine (pH 9.5) was used as the starting buffer and Polybuffer 96 (pH 7.0) (Pharmacia) as the elution buffer. For chromatofocusing of the native SOD, 25 m*M* histidine buffer (pH 6.2) was used as the starting buffer and Polybuffer 74 (pH 4.0) as the elution buffer. For chromatofocusing of recombinant SOD, 25 m*M* histidine buffer (pH 6.2) was used as the starting buffer and Polybuffer 74 (pH 5.0) as the elution buffer. The buffers were prepared according the manufacturer's recommendations (Pharmacia). In all experiments the gradient volume was at least 10 total column volumes. A linear gradient was applied.

Additionally, the FPLC system was equiped with a pH ion monitor (LKB, Bromma, Sweden) to record the pH gradient during elution.

Isoelectric focusing

IEF was performed in Immobiline gels. At low pH, ready-to-use Immobiline dry plates (Pharmacia LKB, Uppsala, Sweden) were used. At high pH, a gel was cast, as described by Wenisch *et al.*[1] using immobilized pH gradients (Immobiline II). The eluted fractions were applied directly to the gel and focused. The gel was stained with Coommassie Blue R 250.

RESULTS

To cover the high and low pH ranges, different proteins were chosen. A human monoclonal antibody exhibiting p*I* between 9.0 and 9.7 was chosen for the basic range and SOD for the acidic range. Highly purified proteins were used as starting

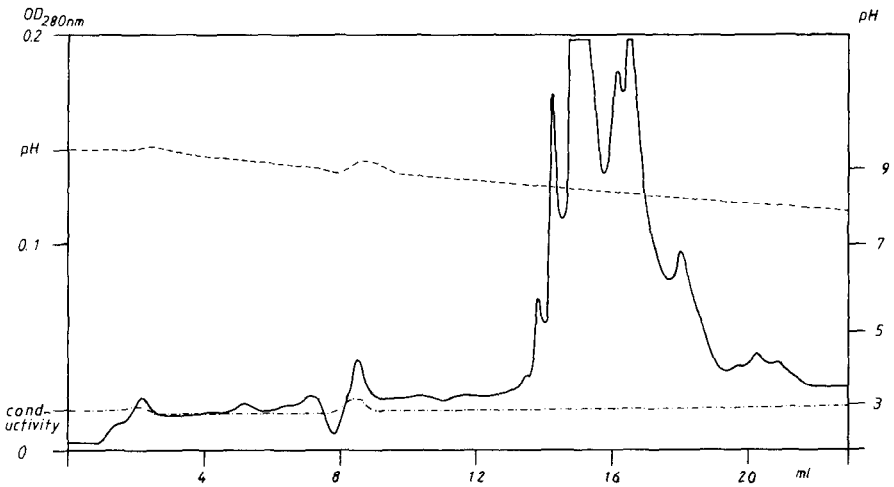

Fig. 1. Separation of isoproteins of a human monoclonal antibody by chromatofocusing on a Mono P column (HR 5/20). Sample: 1 mg of lyophilized monoclonal antibody dissolved in 500 μl of equilibration buffer and loaded onto a 3.4-ml column. Elution was effected with a linear pH gradient from 9.5 to 7.0 at a flow-rate of 0.1 ml/min.

material. A purity of more than 99% was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and silver staining. The highly purified material was chromatographed in a volatile buffer (10 m*M* ammonium formate, pH 8.0) on Sephadex G-25 and lyophilized. The lyophilized material was dissolved in equilibration buffer at a concentration of 4 mg/ml. The sample was injected into the equili-

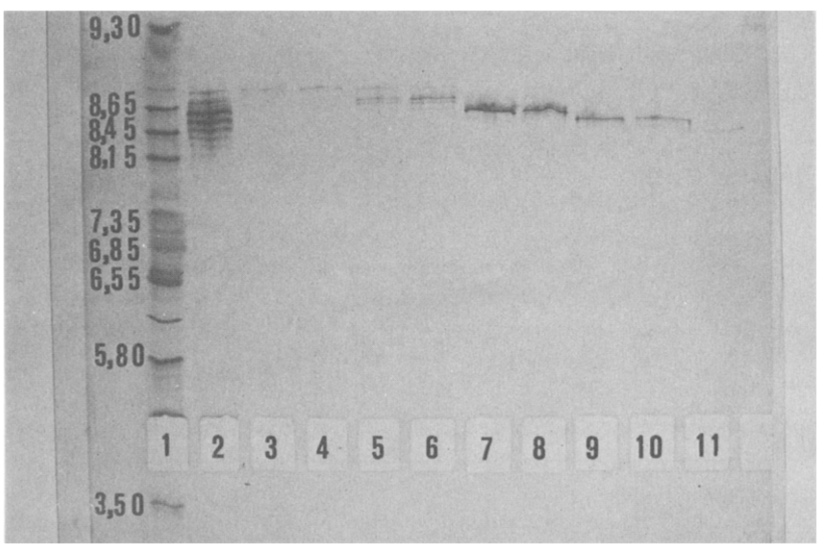

Fig. 2. Isoelectric focusing of the fractions in Fig. 1. Samples: lane 1 = p*I* marker 3–10; lane 2 = starting material (human monoclonal antibody); lanes 3–11 = isoprotein fractions.

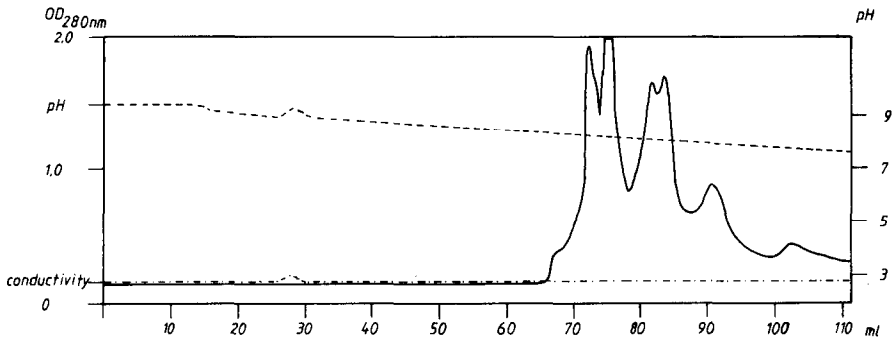

Fig. 3. Separation of isoproteins in a human monoclonal antibody by chromatofocusing on a Mono P column (HR 10/30). Sample: 10 mg of lyophilized human monoclonal antibody dissolved in 2 ml of equilibration buffer and loaded onto a 23-ml column. Elution was effected with a linear pH gradient from 9.5 to 7.0 at a flow-rate of 1 ml/min.

brated and prefocused column. To obtain optimum resolution the linear pH gradient should last for 10–15 column volumes. The isoproteins were eluted by the pH gradient according to their p*I* values. The formation of the gradient was observed with an on-line pH monitor. The peaks were fractionated and the separation was checked by IEF on immobilized pH gradients.

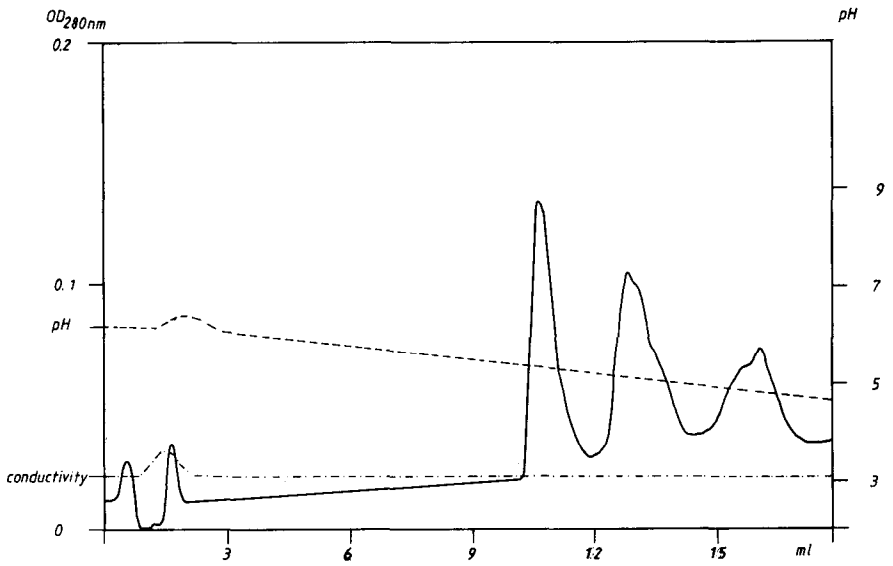

Fig. 4. Separation of isoproteins in human SOD from erythrocytes by chromatofocusing on a Mono P column (HR 5/20). Sample: 1 mg of lyophilized human SOD from erythrocytes dissolved in 500 µl of equilibration buffer and loaded onto a 3.4-ml column. Elution was effected with a linear pH gradient from 6.2 to 4.0 at a flow-rate of 0.1 ml/min.

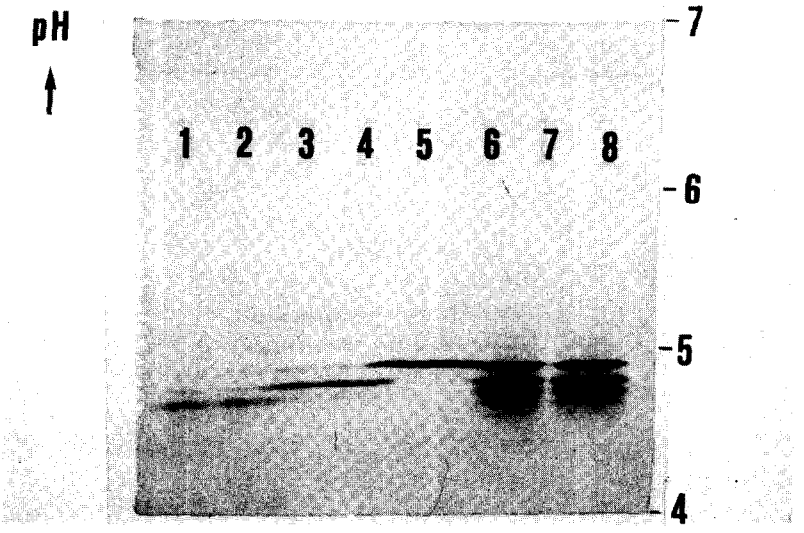

Fig. 5. Isoelectric focusing of the fractions in Fig. 4. Samples: lanes 1–6 = isoprotein fractions; lanes 7 and 8 = starting material (human SOD from erythrocytes).

Focusing of the human monoclonal antibody

The human monoclonal antibody was chromatofocused on two columns, an analytical column and a preparative column. The isoproteins could be resolved on both columns (Figs. 1–3). The number of peaks corresponded to the protein bands in IEF. As a baseline separation could not be obtained, a few fractions were contaminat-

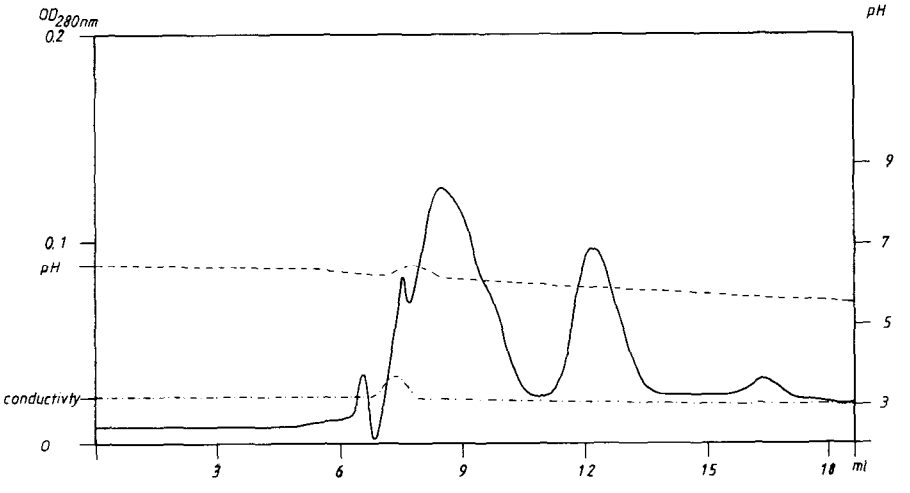

Fig. 6. Separation of isoproteins of human recombinant SOD by chromatofocusing on a Mono P column (HR 5/20). Sample: 1 mg of recombinant human SOD dissolved in 500 μl of equilibration buffer and loaded onto a 3.4-ml column. Elution was effected with a linear pH gradient from 6.2 to 5.0 at a flow-rate of 0.1 ml/min.

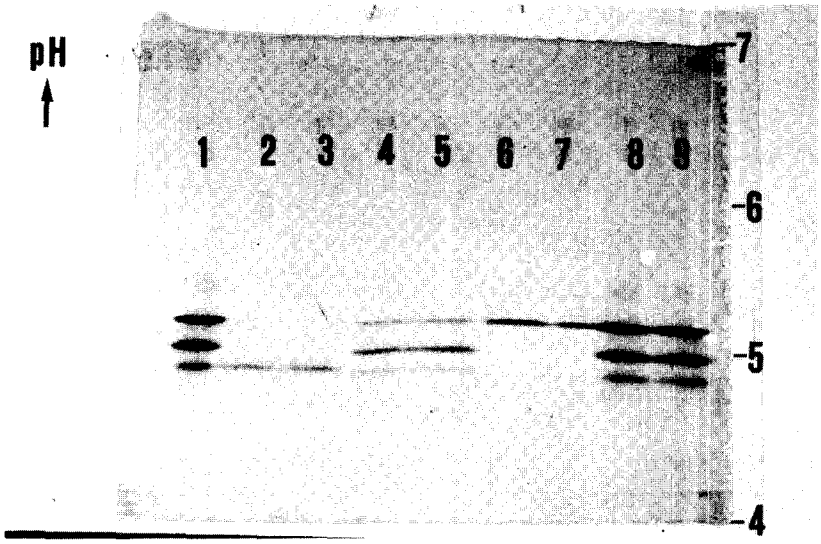

Fig. 7. Isoelectric focusing of the fractions in Fig. 6. Samples: lanes 1, 8 and 9 = starting material (recombinant human SOD); lanes 2–7 = isoprotein fractions.

ed with neighbouring isoproteins. However, the ratio of isoproteins in the antibody samples could easily be determined from the chromatogram.

Focusing of the SOD

Human SOD from erythrocytes and recombinant human SOD were chromatofocused on an analytical column. The native and recombinant SOD differ in the first amino acid, the native enzyme being acetylated.[9] This difference results in a p*I* difference of *ca.* 0.5 pH unit. Both enzymes could be separated into the three isoenzymes and a baseline separation could be achieved (Figs. 4 and 5). IEF also indicated that the separation was nearly complete (Figs. 6 and 7). Arai *et al.*[4] observed this heterogeneity in a highly purified SOD sample by slab gel IEF.

DISCUSSION

Regulations for recombinant proteins and proteins from transformed animal cells to be used as injectable therapeutics require extensive quality control. The potency, purity, identity and consistency must be proved[10,11]. The isoprotein pattern is a valuable method for testing the consistency of different production lots. Generally, IEF is used for this purpose, but there is no exact correlation between concentration and intensity of the stained protein. Therefore, an exact quantitative comparison of different lots is not possible. In qualitative comparisons, chromatographic differences in band patterns can easily be detected. There is an exact relationship between protein concentration in the eluent and detector response. Using various statistical models, the areas of partially overlapping peaks can be calculated with acceptable accuracy. A quantitative measurement of the isoprotein composition of a protein displaying microheterogeneity can be carried out.

For further investigations, such as peptide mapping or the determination of carbohydrate moieties, the separation of the isoproteins is essential. In this paper an attempt has been made to show that chromatofocusing could be an alternative to conventional preparative IEF[12–14]. The various isoproteins could be partially separated, as shown by IEF.

ACKNOWLEDGEMENTS

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