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Purification of human adenosine deaminase for the preparation of a reference material

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Abstract

The goal was to optimise a purification procedure of adenosine deaminase from human erythrocytes for the preparation of a European Reference Material. Adenosine deaminase was purified from human erythrocytes with a specific activity of 4.46 μkat/mg of protein and a catalytic concentration of 133 μkat/l. The isolation and purification procedure involved ion-exchange chromatography (STREAMLINE™ DEAE), and two purine riboside affinity chromatographies. The purified enzyme exhibits a single band in SDS–PAGE with a molecular weight of 41 600 g/mol, and three bands in PAGE, isoelectric focusing and two-dimensional electrophoresis with p*I* 4.7, 4.85 and 5.0.

Keywords: Purification; Enzymes; Adenosine deaminase

1. Introduction

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4) is a metalloenzyme involved in the salvage of purine nucleosides and catalyzes the hydrolysis of adenosine or deoxyadenosine to inosine or deoxyinosine and ammonia [1]. In human serum adenosine deaminase activity shows three molecular forms: a monomeric form of 35 000 g/mol (ADA 1); a tetrameric form of 280 000 g/mol formed by the combination of two monomers of ADA 1 and a dimeric glycoprotein named combining protein; and a third form of 100 000 g/mol (ADA 2). These forms differ in kinetic and immunological properties [2].

It has been reported that abnormalities of the adenosine deaminase activity are related to the development of certain genetic diseases [3]. Also, alterations of adenosine deaminase activity in other diseases, such as tuberculosis [4], AIDS [5] and typhoid fever [6], have been described. Measurement of adenosine deaminase catalytic concentration in pleural fluids is an important diagnostic tool for tuberculosis.

Adenosine deaminase is widely distributed in almost all tissues. It is a genetically polymorphic enzyme which appears to be controlled by a single locus [7]. It has been suggested that adenosine deaminase 1 is controlled by two allelic genes [8].

Several methods for adenosine deaminase purification have been described in the literature [9–11].

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Those that achieve higher specific activities require the use of affinity chromatography with specific antibodies against adenosine deaminase as proposed by Daddona and Kelley [9], or with purine riboside, an adenosine deaminase inhibitor, as described by Aran et al. [10].

The purpose of this work is to describe a new purification procedure for adenosine deaminase 1 from human erythrocytes using a STREAMLINE™ DEAE expanded bed absorption and two affinity chromatographies with the inhibitor purine riboside. The purified adenosine deaminase 1 will be used for the preparation of a European Reference Material.

2. Experimental

2.1. Chemicals

Adenosine, deoxyadenosine, 2-oxoglutarate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium salt (MTT), phenazine methosulphate, nucleoside phosphorylase, purine riboside (nebularine) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) were purchased from Sigma (St. Louis, MO, USA). NADH and ADP were from Boehringer Mannheim (Mannheim, Germany). Glutamate dehydrogenase and xanthine oxidase were from Biozyme (Blaenovon, UK). STREAMLINE™ DEAE, epoxy-activated Sepharose CL6B, and silver staining reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-human adenosine deaminase antibody and sheep anti-rabbit IgG were purchased from Labgen (Frankfurt, Germany). All other reagents used were the highest grade commercially available.

2.2. Instruments and equipment

The FPLC system was composed of a FRAC-200 fraction collector, pump-P50, UV and conductivity detector controller and columns: XK-26 and STREAMLINE™ 25 (Amersham). Enzyme assays were carried out in a COBAS-MIRA analyzer (Roche, Basel, Switzerland). The electrophoresis system was from Amersham. Stirred cells and disc membranes PM10 were from Amicon (Beverly, USA).

2.3. Adenosine deaminase assay

Adenosine deaminase catalytic concentration was measured using the method described by Ellis and Goldberg [12] and optimized by Bota et al. [13]. The ammonia formed by the catalytic activity of adenosine deaminase was coupled to the reaction catalysed by glutamate dehydrogenase. The rate of consumption of NADH is measured at 340 nm and 37°C. The concentrations of the reagents and the settings for measuring adenosine deaminase catalytic concentration are showed in Table 1.

2.4. Protein determination

Protein concentration was determined by the method described by Bradford [14] with Coomassie Brilliant Blue as reagent and bovine serum albumin as standard; and also following its absorbance at 280 nm.

2.5. STREAMLINE ™ chromatography

The STREAMLINE™ methodology is an expanded bed absorption specially designed by capturing protein originating of unclarified sample, crude extract, fermentation, cell culture, etc. Target proteins are captured on the adsorbent while cell debris and cell contaminants pass through unhindered. Flow is then reversed, and target proteins are desorbed by an elution buffer as in conventional packed bed (Fig. 1).

Table 1
Final concentrations of the reagents for measuring adenosine deaminase catalytic concentration optimized

Phosphate buffer	200 mmol/l
pH	7.2
Adenosine	20.0 mmol/l
2-Oxoglutarate	14.0 mmol/l
NADH	0.33 mmol/l
Glutamate dehydrogenase	950 μkat/l
ADP	2.50 mmol/l
Measurement time	300 s
Incubation time	325 s

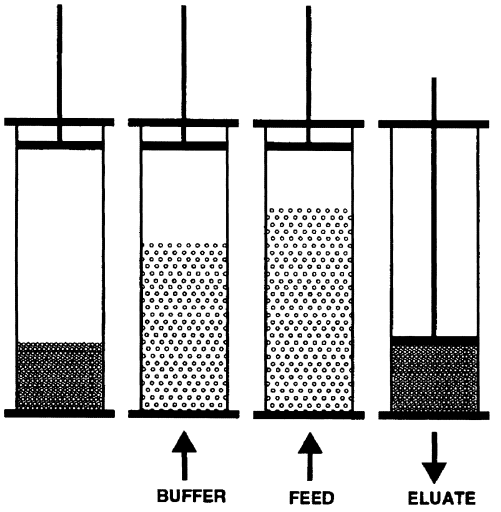

Fig. 1. STREAMLINE technology: normal state, buffer application, feed application, reverse flow and elution.

2.6. Affinity chromatography preparation

The ligand purine riboside was coupled to epoxy-activated Sepharose 6B following the method described by Aran et al. [10]. The activation was made by incubating, at 37°C, 30 g of epoxy-activated Sepharose 6B resin with 200 ml of 25 mmol/l purine riboside, in 0.1 mol/l $NaHCO_3$ (pH 11.2). In essence, this method is similar to that the employed by Schrader et al. [11] for coupling adenosine to the epoxy-activated resin. Based on the amount of purine riboside removed (measured by its absorbance at 260 nm) from the solution after 24-h coupling period, the yields of purine riboside bound per gram of epoxy-activated Sepharose 6B was 30 μmol. The affinity gel was stored in 25 mmol/l Tris–HCl, pH 7.4.

2.7. Enzyme purification

2.7.1. Preparation of the hemolysate

Outdated packed erythrocytes were obtained from healthy subjects from the blood bank of Hospital de Sant Pau (Barcelona). Sera of the subjects were free of hepatitis B antigen and HIV antibodies. The erythrocytes were washed twice with equal volume of 0.15 mol/l NaCl and the packed cells obtained after centrifugation at 9500 g 15 min at 4°C, were lysed with 15 mmol/l phosphate buffer, pH 7.0, and mixed overnight. The disrupted cells were sonicated (during 5 min at intervals of 30 s).

2.7.2. STREAMLINE™ DEAE chromatography

The hemolysate was directly pumped upward on 130 ml (30 cm) of STREAMLINE™ DEAE equilibrated with 15 mmol/l phosphate buffer, pH 7.0, and expanded to 390 ml (90 cm) on a STREAMLINE™ 25 column at a flow-rate of 22 ml/min. Adenosine deaminase was retained by the adsorbent. After washing with 1000 ml of buffer, the enzyme was eluted at a flow-rate at 8 ml/min with 2 vol of a linear NaCl gradient from 0 to 0.2 mol/l in the same buffer. Fractions containinng adenosine deaminase activity were pooled, concentrated by ultrafiltration using a disc membranes PM10 and dialysed.

2.7.3. Affinity chromatographies

The dialysate was applied to purine riboside Sepharose column (80 ml) equilibrated with 25 mmol/l Tris–HCl buffer, pH 7.4, at a flow-rate of 3 ml/min. After washing with 400 ml of buffer the retained enzyme was eluted with 100 ml of linear NaCl gradient from 0–0.1 mol/l. The active fractions were pooled, concentrated by ultrafiltration, dialysed and applied again to the purine riboside Sepharose column, previously washed and re-equilibrated. The second affinity chromatography was run under the same conditions described for the first.

2.8. Electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis in the presence (SDS–PAGE) or absence (PAGE) of sodium dodecyl sulphate using a discontinuous buffer system was performed according to Laemli and co-worker [15,16]. Electrophoresis were performed in a resolving and stacking gel of 10 and 4% polyacrylamide, respectively. After electrophoresis, protein bands were silver stained or stained with Coomassie. The molecular mass of adenosine deaminase was calculated on the basis of a linear relationship of the logarithm molecular mass to the R_f in a SDS–PAGE.

Proteins separated by SDS–PAGE or PAGE were electroblotted on to PVDF membrane. The detection was made with a rabbit anti-human adenosine deaminase and sheep anti-rabbit IgG.

Electrophoresis with adenosine deaminase activity staining was carried out at 150 V and 15 mA for 3 h

with refrigeration at 4°C. The activity was made visible using a modification of the adenosine deaminase reaction [17,18]. The staining reaction mixture was as follows: 20 mg adenosine, 0.026 μkat nucleoside phosphorylase, 2.6 mkat xanthine oxidase, 10 mg MTT, and <1 mg phenazine methosulphate in 50 ml phosphate buffer 250 mmol/l, pH 7.4.

3. Results and discussion

Adenosine deaminase was purified from human erythrocytes by three steps purification process involving anionic exchange chromatography in an expanded bed adsorption and two affinity chromatographies.

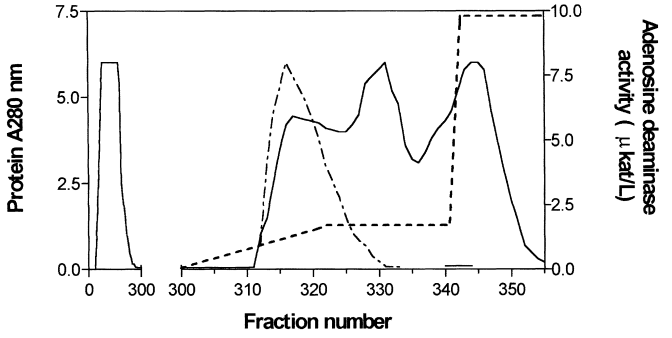

Fig. 2. Chromatography on STREAMLINE ™ DEAE at pH 7.0. Protein concentration (mg/ml) by absorbance at 280 nm(———), adenosine deaminase catalytic concentration activity (– · –), and sodium chloride gradient (- - -).

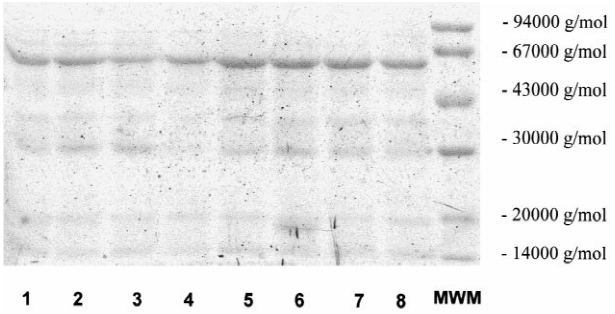

Fig. 3. SDS–PAGE of pre-purified adenosine deaminase from eight STREAMLINE pools: lanes 1–8, molecular weight markers (MWM).

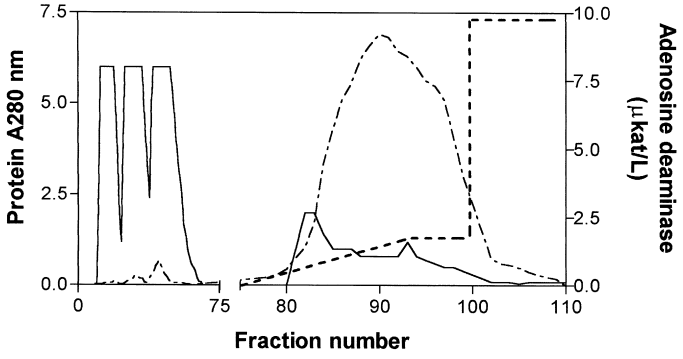

Fig. 4. Chromatography on purine riboside sepharose at pH 7.4. Protein concentration (mg/ml) by absorbance at 280 nm (———), adenosine deaminase catalytic concentration (– · –), and sodium chloride gradient (- - -).

Due to the low adenosine deaminase activity present in human erythrocytes it was necessary to develop a first step of purification which allowed to process a large volume of sample. The use of STREAMLINE™ DEAE chromatography proved to be a powerful tool to partially purify human adenosine deaminase directly from unclarified lysed erythrocytes solution. It allows direct capture of the enzyme, without the need for prior clarification, resulting in a reduced number of processing steps. It offers also the possibility to process a large volume of solution reducing the manipulation of blood, which is especially critical in human samples because of the possible risk of infectivity.

3.1. First step capture: STREAMLINE™ DEAE chromatography

The hemolysate resulting from 7 l of packed erythrocytes was directly pumped upward, using STREAMLINE™ DEAE expanded on a STREAMLINE™ 25 column. The process was repeated 30 times applying 310 ml of hemolysate each time. Adenosine deaminase was retained by the gel and eluted as a simple peak at 70 mmol/l NaCl. Fractions containing adenosine deaminase activity were pooled and concentrated by ultrafiltration until a final volume of 20 ml and stored at 4°C. All concentrates (600 ml) were pooled, concentrated and dialysed overnight against 20 l of 25 mmol/l Tris–HCl buffer, pH 7.4. Fig. 2 shows a typical chromatographic profile on STREAMLINE DEAE.

The protein pattern as well as adenosine deaminase specific activity (from 2 to 4 μkat/g of protein) were the same in all STREAMLINE™ chromatographies, as shown in Fig. 3. STREAMLINE™ DEAE allowed to eliminate the major part of albumin and all of the haemoglobin, which were the major contaminants of adenosine deaminase in human erythrocytes.

At the end of the process the adsorbent had the same performance as the first time. After each 10 chromatographies a little cleaning-in-place process was carried out, as indicated by the manufacturer. This was a great advantage because the haemoglobin of erythrocytes stained other adsorbents that we tested before STREAMLINE™, such as CM-Sepharose CL6B, DEAE Sephacel, and CM-Sepharose fast

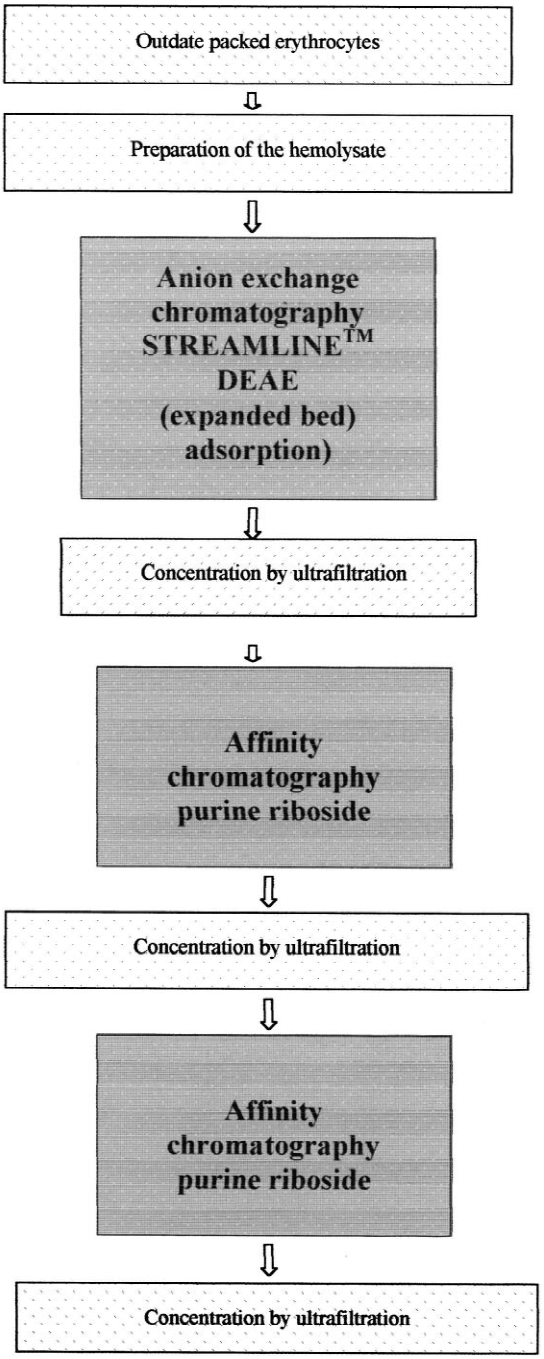

Fig. 5. Flow chart of the adenosine deaminase purification procedure.

flow. Cleaning sessions were necessary more frequently, and finally the adsorbents lost their binding capacity.

3.2. Second and third step polishing: affinity chromatographies

The dialysate of the previous step (125 ml, 4×) was applied to a purine riboside Sepharose column as described in Section 2. Adenosine deaminase eluted as single peak, as shown in Fig. 4. The active fractions of the four chromatographies were pooled and concentrated by ultrafiltration. The concentrate was dialysed in the same stirred cell and applied again to the purine riboside Sepharose column. Adenosine deaminase, eluted as a single peak, was pooled, concentrated by ultrafiltration to a final volume of 24 ml and stored at −80°C.

In this step we compared different chromatographies: gel filtration (Sephacryl-S200 SF); ion-exchange (CM-Sepharose); and affinity chroma-

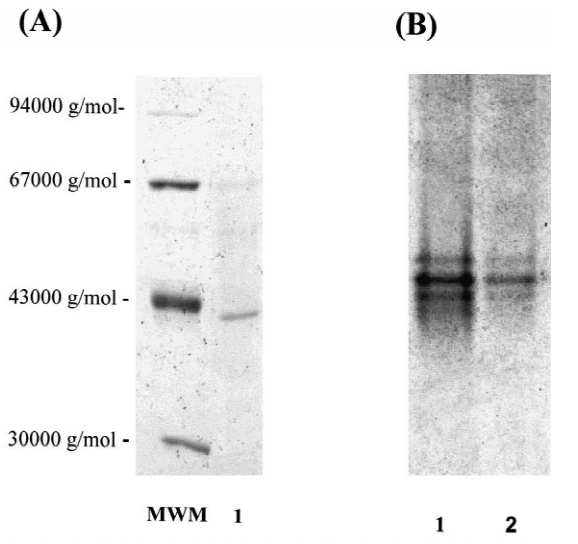

Fig. 6. (A) SDS–PAGE of purified adenosine deaminase. MWM, molecular weight markers; lane 1, purified adenosine deaminase (0.8 μg). (B) PAGE of purified adenosine deaminase: lane 1, 0.8 μg; lane 2, 0.4 μg.

Table 2
Summary of the purification results starting from 7 l of packed erythrocytes

Stage	Protein (mg)	Catalytic activity (μkat/l)	Specific activity (μkat/mg)	Recovery (%)	Purification (fold)
STREAMLINE™ DEAE	3046	1.8	0.0035	100	1
Purine riboside 1	6.6	8.7	0.91	61	323
Purine riboside 2	0.7	27.5	4.46	32	1274

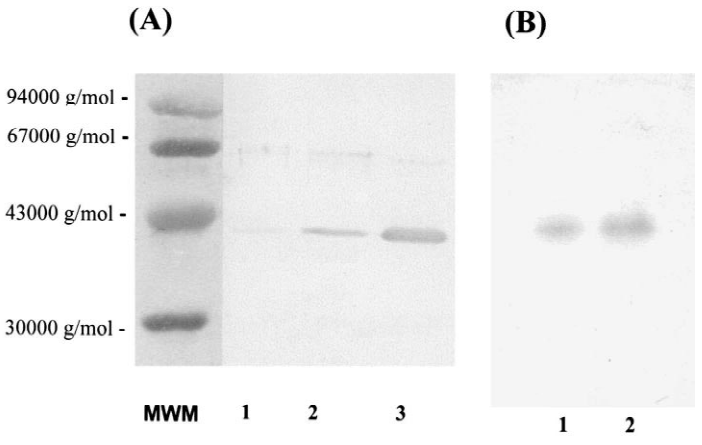

Fig. 7. (A) SDS–PAGE Western blotting: MWM, molecular weight markers; lane 1, STREAMLINE; lane 2, purine riboside 1; lane 3, purine riboside 2. (B) PAGE Western blotting: lane 1, 0.8 μg; lane 2, 0.4 μg of purified enzyme were applied.

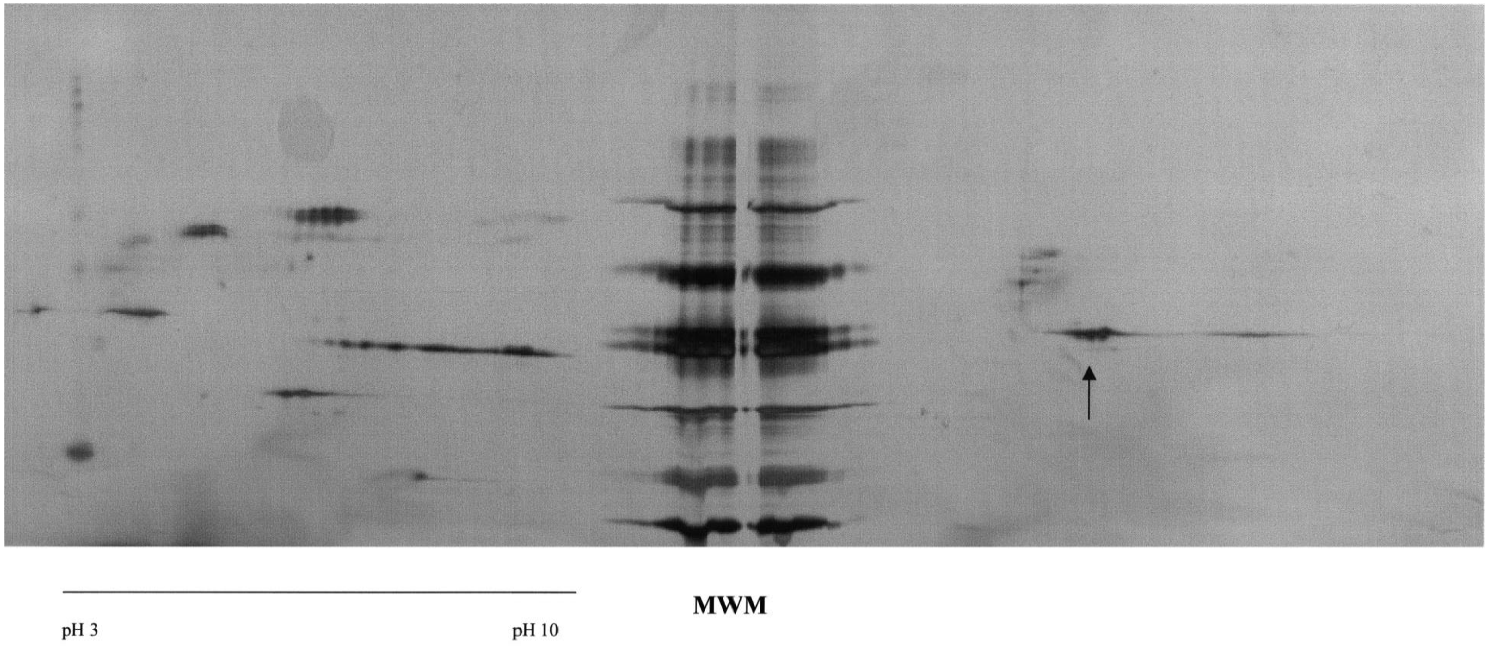

Fig. 8. Two-dimensional electrophoresis: adenosine deaminase, molecular weight markers, 94 000–14 000 g/mol; pH range, 3–10. Adenosine deaminase is marked with an arrow.

tography (purine riboside). The best results were obtained with affinity chromatography as described by different authors [9,17]. This chromatography has the advantage that the deamination of the purine riboside by the action of the enzyme (the compound has no amino group in the C6 position) is impossible. Due to this change, of an amino group by hydrogen, the substrate is altered to an inhibitor and thus the affinity column can be re-used many times.

A summary of the purification results obtained are shown in Table 2, as well as a flow chart of the purification process in Fig. 5. About 0.7 mg of purified adenosine deaminase were obtained from 7 l of erythrocytes with a catalytic activity of 4.46 μkat/mg of protein and a catalytic concentration of 133 μkat/l.

3.3. Molecular characterisation of the purified enzyme

The purity of the preparation was checked by SDS–PAGE, showing a single band with a relative molecular mass of 41 600 g/mol (Fig. 6A), that was identified as adenosine deaminase by Western blotting with a monoclonal antibody against human adenosine deaminase (Fig. 7A). The purified enzyme was also analysed by PAGE and showed three bands (Fig. 6B). Western blotting of PAGE using the same monoclonal antibody showed a single diffusion line (Fig. 7B). Two-dimensional electrophoresis of the purified enzyme showed one band of 41 000 g/mol and three spots with differents p*I* values: 4.7, 4.85 and 5.0 (Fig. 8).

PAGE with adenosine deaminase reaction staining showed one band in the same position (figure not shown).

3.4. Kinetic characterisation of the purified enzyme

The apparent Michaelis constant (K_m) for the substrates adenosine and deoxyadenosine, the inhibition constant (K_i) for the inhibitors EHNA and purine riboside and the optimum pH have been determined. The values are shown in Table 3 and they are very similar to those reported in the literature [19,20].

In conclusion, the results of adenosine deaminase purification, its level of purity and its molecular and kinetic characterisation were considered suitable for the preparation of a European Reference Material for clinical laboratory use.

Table 3
Kinetic constants of purified adenosine deaminase

Adenosine	K_m	0.06±0.003 mmol/l
	V_{max}	150 μmol/min
	K_i (EHNA)	0.03±0.014 μmol/l
	K_i (nebularine)	0.03±0.018 mmol/l
	Optimum pH	7.15
Deoxyadenosine	K_m	0.09±0.005 mmol/l
	V_{max}	96 μmol/min
	K_i (EHNA)	0.04±0.055 μmol/l
	K_i (nebularine)	0.08±0.021 mmol/l
	Optimum pH	7.25

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