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Simple purification procedure for human prostatic kallikrein hK2 in its active form

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Abstract

Kallikrein hK2 is a new potential marker of prostate cancer. It is the last member of the human kallikrein gene family to be isolated. We propose a simple purification procedure permitting us to obtain the active form of hK2 starting from human seminal plasma and using commonly available chromatography matrices. In contrast to recently published papers, this procedure is carried out without any immunoaffinity chromatography step and without the need for any antibody to follow the purification. Furthermore, it does not require any recombinant DNA technology nor sophisticated instruments. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Kallikrein hK2; Proteins

1. Introduction

Human prostatic kallikrein hK2 is a new promising serum and tissue marker for prostatic cancer [1–3]. The protein also has enzymatic properties which could favor prostatic cancer cell progression [4,5]. For these reasons, there is a growing interest to determine its concentration in biological fluids and to study its properties. However, the hK2 protein needed for these studies is not readily available in spite of the fact that purification procedures have been described both for native hK2 [6] as well as for the recombinant protein [7,8]. In fact, in two of these procedures [6,7], an immunoaffinity chromatography step with specific anti-hK2 monoclonal antibodies (MAbs) was needed while in the other case [8], a recombinant hK2 protein was produced with the baculovirus system and purified by chromatographic procedures using a polyclonal anti-PSA antibody to detect hK2 since PSA and hK2 have 78% amino acid identity. In the present paper, we describe a simple purification procedure for hK2 from human seminal plasma without the use of any antibody and/or recombinant DNA technology.

2. Materials and methods

2.1. Materials

Dialysis membranes Spectra Por 2 (12–14 kDa cut-off) were purchased from Fisher Chemical Company, Montréal, Canada. The chromatography matrices DEAE-Sepharose CL-4B, CM-Sepharose CL-4B and Benzamidine-Sepharose CL-4B were obtained from Pharmacia, Baie d'Urfé, Canada. The

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kallikrein substrate Pro-Phe-Arg-pNA was purchased from Sigma–Aldrich Canada Ltd, Oakville, Canada.

2.2. Seminal plasma

Sperm samples were obtained from the CHUL Andrology Laboratory, directed by one of the authors (RRT). The specimens originated both from normal men and from infertile patients. Each subject signed an informed consent form specifying that he agreed on the use of the anonymous leftover samples for research purposes on seminal plasma proteins.

2.3. Purification procedure for hK2

The seminal plasma was obtained by centrifugation of sperm samples at 10 000 *g′* for 10 min. A total volume of 100 ml of seminal plasma was dialyzed twice for 24 h against 2 l of 50 m*M* Tris-HCl buffer pH 7.0 in Spectra/Por 2 tubing (12–14 kDa cut-off). The content of the dialysis tube was centrifuged at 8 000 *g′* for 20 min to remove precipitated material.

The second step was the passage of the dialyzed plasma (140 ml) through a column of 20 ml of DEAE-Sepharose CL-4B made in a 20 ml plastic syringe. During the deposition of the material on the column, all the flow-through volume was collected as well as an additional 75 ml volume representing the washing of the column with 50 m*M* Tris-HCl buffer. All these flow-through fractions (220 ml) were adjusted to pH 6.3 with 1 *M* HCl and mixed with 0.8 volume (170 ml) of 10 m*M* sodium phosphate buffer pH 6.3. That mixture was centrifuged at 8 000 *g′* for 15 min and the supernatant was kept for further analysis.

The third step was the column chromatography of DEAE-Sepharose flow-through fractions on a 20 ml column of CM-Sepharose CL-4B. That column was also made in a 20 ml plastic syringe. All the flow-through fractions were discarded as well as the washings with 30 ml of buffer. The column was then successively eluted with 200 ml of 10 m*M* sodium phosphate buffer pH 6.3 containing 0.1 *M* NaCl and then with 40 ml of the same buffer containing 0.5 *M* NaCl. That last 40 ml fraction which contained the hK2-protein C inhibitor (PCI) complex was dialyzed three times for 8 to 20 h with phosphate buffered saline (PBS).

The fourth step was the decomplexation of the hK2-PCI complex at alkaline pH as previously described [6]. Briefly, the dialyzed material of the 0.5 *M* NaCl fraction of the CM-Sepharose chromatography was diluted with 0.5 vol of 100 m*M* sodium phosphate buffer at pH 11.4. That buffer was prepared freshly with 100 m*M* Na_2HPO and adjusted to pH 11.4 with 1 *M* NaOH. The incubation with the hK2-PCI complex was carried out at room temperature for 60 min. The reaction was stopped by bringing the pH to 8.1 with 1 *M* HCl.

The last step was affinity chromatography on a 5 ml column of Benzamidine Sepharose CL-4B made with a 5 ml plastic syringe. The column was first equilibrated at room temperature with 50 m*M* Tris-HCl buffer pH 8.0 containing 150 m*M* NaCl. The decomplexed material from the previous step was passed through the column which was further washed with the equilibration buffer until the optical density reached negligible values (0–0.002 O.D. units). The specifically adsorbed proteins were eluted with 150 m*M* NaCl containing 0.003 *M* HCl. Fractions of 1.0 ml were collected. The highest proportion of the eluted hK2 was found in fraction 6 (71%) with smaller amounts in fractions 5 (8%) and 7 (19%). Immediately after the elution, fractions were neutralized with 10 μl of 0.9 *M* Tris-HCl buffer pH 7.5 containing 1.8% NP-40.

2.4. Characterization of purified hK2

The affinity-purified hK2 was characterized by SDS gel electrophoresis according to Laemmli [9] and by Western blot analysis using specific MAbs against hK2 (MAb 9D5) [10] and the ECL detection system of Amersham, Oakville, Canada. The enzymatic activity of the purified hK2 preparations was determined with Pro–Phe–Arg–pNA, a very good substrate of hK2 [11]. The assays were performed in 50 m*M* Tris-HCl buffer at pH 8.0 containing O.1 m*M* of substrate. An absorbance increase at 405 nm was recorded. The activities observed with three different purification runs were compared to those of three preparations obtained by our previous procedure [6].

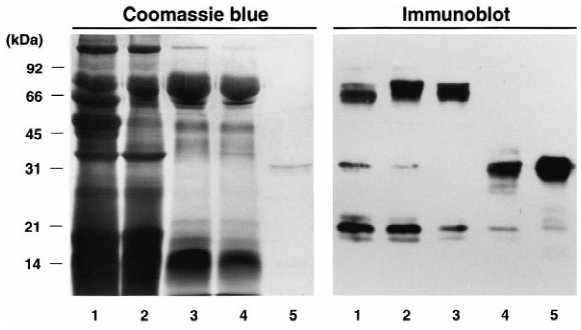

Fig. 1. Characterization by SDS gel electrophoresis (Coomassie blue) and Western blotting (Immunoblot) with MAb 9D5 of the seminal plasma proteins at different steps of the hK2 purification procedure. Lane 1: seminal plasma; lane 2: flow-through fractions of the DEAE-Sepharose column; lane 3: proteins eluted from the CM-Sepharose column with 0.5 *M* NaCl; lane 4: same material as in lane 3 but incubated at pH 11.4 (decomplexation step); lane 5: proteins eluted from the Benzaznidine–Sepharose column with 0.003 *M* HC1. For the Coonlassie blue staining experiment as well as the immunoblot, the amount of protein deposited in lanes 1 to 4 represented the same proportion of the starting material. In lane 5 of the Coomassie blue stained gel, the amount of protein was adjusted to approximately 0.5 μg while in lane 5 of the immunoblot, it was approximately 15 ng.

3. Results

The whole purification procedure required approximately one week. Fig. 1 summarizes the behaviour of kallikrein hK2 and its molecular forms during the various chromatographic steps as determined by Western blot analysis with MAb 9D5. It shows that the starting material contains three types of hK2 molecular forms: hK2-PCI complexes (60–75 kDa), free hK2 (31 kDa) and hK2 nicked forms (22 kDa). All of these forms are recovered in the DEAE–Sepharose flow-through fractions. After adsorption on a CM–Sepharose column, the 60–75 kDa complex and the 22 kDa nicked forms are eluted with 0.5 *M* NaCl (Fig. 1, lane 3). Subsequently to an incubation at pH 11.4 (decomplexation step), the hK2-PCI complexes are no longer present (Fig. 1, lane 4). They have been transformed into free hK2 either as single-chain or as nicked forms. The affinity-chromatography step on Benzamidine–Sepharose yielded a 31 kDa doublet with very little inactive 22 kDa nicked forms (seen only on the immunoblot). The amount of contaminating proteins stained with Coomassie blue was negligible. Therefore, on the basis of SDS gel electrophoretic pattern, the hK2 preparation in this study appeared similar to that obtained previously by immunoaffinity chromatography [6].

As far as yield is concerned, we recovered 10 to 20 μg of hK2 from 100 ml of seminal plasma. That result indicates that the overall recovery was approximately 10% of total seminal plasma hK2, whose concentration is estimated to be 2 μg per ml by ELISA (unpublished results). A large proportion of the losses occurred during the Benzantidine–Sepharose chromatography step, indicating that, after the decomplexation step, hK2 was mostly inactive either because of the presence of internal cleavages or because of chemical denaturation during the purification procedures. Nevertheless, the remaining active hK2 was efficiently recovered during the Benzaznidine–Sepharose step as evidenced by the fact that 60% of the Pro–Phe–Arg–pNA hydrolytic activity applied to the column could be recovered during the elution at low pH.

To ascertain the quality of three hK2 preparations obtained in this study, we compared their enzymatic activity to that of three other preparations obtained previously using immunoaffinity-chromatography and C_{18} reversed-phase HPLC [6]. We found that the hydrolysis of Pro–Phe–Arg–pNA was 3.0 times as high as the hydrolysis observed with the immunoaffinity purified material which included a large proportion of inactive enzyme.

4. Discussion

This study shows that it is relatively easy to purify the enzymatically active form of kallikrein hK2 from human seminal plasma, in spite of its low concentration in that fluid and of its occurrence as an inactive molecular complex with PCI in whole seminal plasma. The overall yield of hK2 may seem low when considering the total hK2 concentration in human seminal plasma. However, it must be mentioned that a large proportion of the active enzyme has been recovered. Furthermore, the yield obtained in the present study (10 to 20 μg $(100\ ml)^{-1}$) was slightly lower than that obtained by our previous procedure (40–50 μg $(100\ ml^{-1})$), which included both the active and the inactive enzyme [6]. The

lower yield was compensated by the higher activity of the preparation. We therefore conclude that the proposed new procedure has several advantages over previously published procedures as it yields the natural protein and does not necessitate sophisticated technology such as recombinant DNA and/or monoclonal antibodies.

This type of hK2 preparation should be useful for the development of new specific monoclonal antibodies. Indeed, recent studies point out the potential utility of hK2 as a marker for prostatic cancer [1–3]. At the same time, these studies indicate the necessity for the development of other hK2-specific antibodies having lower cross-reactivity with PSA and having higher sensitivities, since hK2 is present at very low concentrations in the serum. All the anti-hK2 antibodies that have been described up to now were obtained by using recombinant proteins as the antigenic preparations. A priori, the native hK2 protein such as is obtained in this study could result in the production of superior antibodies for immunoassay procedures. Furthermore, this preparation should be an asset for enzymatic studies when compared to recombinant prohK2 proteins that need to be activated by trypsin [7] or by refolding in harsh conditions [12].

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