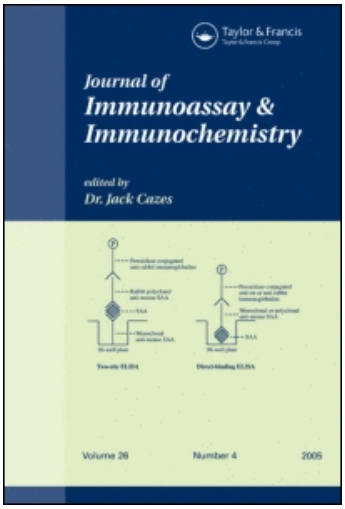

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MONOCLONAL ANTIBODIES AGAINST HUMAN ENDOTHELIN-CONVERTING ENZYME-1

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ABSTRACT

Endothelin-converting enzyme-1 (ECE-1) is a membrane-bound metalloprotease which specifically converts the inactive precursor big-endothelin-1 (big ET-1) to the vasoactive endothelin-1 (ET-1). Six different mouse hybridoma cell lines have been generated secreting monoclonal antibodies specific to human ECE-1. These antibodies have been proven useful in a fast and efficient one-step purification of membrane-bound ECE-1 as well as of artificial soluble ECE-1 by immunoaffinity chromatography. The antibodies are suitable for a quantification of ECE-1 in solution by a sandwich-ELISA and for the immunohistochemical detection of ECE-1 in the cell membrane.

(KEY WORDS: monoclonal antibody, endothelin-converting enzyme-1, ELISA, affinity chromatography)

INTRODUCTION

Endothelin-1 (ET-1) is a potent vasoconstrictive and mitogenic peptide of 21 amino acids which was originally isolated from the supernatant of porcine endothelial cell culture (1). ET-1 plays a role in vascular smooth muscle cell growth. Elevated levels are associated with several pathophysiological conditions and ET-1 is possibly involved in congestive heart failure, acute and chronic renal failure, cerebral ischemia and vasospasm, pulmonary hypertension, endotoxic shock, asthma and subarachnoid hemorrhage. Furthermore ET-1 seems to play a role in atherosclerosis and restenosis after PTCA (2-11).

ET-1 is generated by proteolytic cleavage of the inactive precursor big ET-1 between Trp^{21} and Val^{22} by the zinc-metalloprotease endothelin-converting enzyme-1 (ECE-1) (12). Due to the involvement of ET-1 in several pathological situations and the fact that ECE-1 is a key enzyme in the biosynthesis of ET-1, ECE-1 is an interesting target for pharmacological intervention. ECE-1 is a membrane-bound, disulphide-linked dimeric glycoprotein (10 possible N-linked glycosylation sites), composed of 2 identical subunits with a MW of about 125 kDa each. The pH optimum of the enzymatic activity is in the neutral range (13-17) and ECE-1 belongs to a class of neutral metalloproteases.

Two related isoforms, ECE-1α and ECE-1β, with minor differences in the cytoplasmic part are identified showing no differences in the enzymatic activity and substrate specificity. The subcellular localization of ECE-1α and ECE-1β and the cellular and tissue distribution seem to be different and are presently investigated.

It is suggested that the transcription of these isoforms might be directed by use of an alternative promotor in the second intron (18-20).

The cDNA of ECE-1 from different species (human, rat, bovine) have been cloned. The comparison reveals a high degree of conservation (13, 14, 17, 21). Furthermore there is a significant structural and sequence homology to neutral endopeptidase 3.4.24.11 (NEP 24.11) and Kell human blood group antigen.

Selective antibodies directed against ECE-1 could help in the identification of the tissue and subcellular localization of ECE-1 by immunohistochemistry. ECE-1 activity has been detected in the lipid fraction of plasma (22). By means of antibodies to human ECE-1 it should be possible to confirm these results and provide data to a better understanding for the physiological / pathophysiological basis of this phenomenon. Therefore one of the purposes of this study was to establish an antibody-based assay to measure ECE-1 in human plasma.

Immunoaffinity chromatography could be a very useful and easy step in the purification of the enzyme.

MATERIALS AND METHODS

ECE-1 Preparation

Recombinant human ECE-1 of transfected CHO-K1 cells was isolated with a purity of >80% as described (14) and used as antigen.

A soluble monomeric form of ECE-1 was generated by fusion of a human ECE-1 cDNA encoding the extracellular part of ECE-1, which contains the catalytic domain in frame with a cDNA of the human t-PA signal sequence. The chimeric cDNA was expressed in CHO-K1 cells and the secreted protein was purified in analogy to the full-length ECE-1.

Immunization and Fusion Procedure

Female NZ white rabbits (Thomae, Germany), about 10 weeks old were immunized 5 times s.c. and i.m. with 200 µg r-hu ECE-1. The polyclonal IgG`s were isolated on protein A glass beads (ProsepA/Bioprocessing).

Female Balb c mice, 6-8 weeks old (Thomae, Germany) were immunized i.p. 5 times with 100 µg ECE-1. The spleens of mice showing a strong antibody response were removed 3 days after the last immunization. The cells were fused with the myeloma cells SP2/0-Ag14 (ATCC CRL1581) and grown in Dulbecco´s modified Eagle medium (DMEM, Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies), HAT (Boehringer Mannheim), 10% (v/v) Condimed (Boehringer Mannheim) and Insulin-Transferrin-Selenite 1:1000 (Boehringer Mannheim).

Positive clones were identified by a direct ELISA respectively by a sandwich-ELISA.

Direct ELISA: coating of the ELISA plate with r-hu-ECE-1 / incubation with hybridoma cell culture supernatant containing the monoclonal antibodies / detection of the mAb´s by incubation with biotinylated anti-mouse Ab, streptavidin-peroxidase complex and tetramethylbenzidine substrate.

Sandwich-ELISA: coating of the ELISA plate with polyclonal rabbit anti-ECE-1 antibody / incubation with r-hu-ECE-1 / incubation with hybridoma cell culture supernatant containing the monoclonal antibodies / detection of the mAb´s by incubation with biotinylated anti-mouse Ab, streptavidin-peroxidase complex and tetramethylbenzidine substrate.

Unspecific antibodies were identified and eliminated by a direct counterscreen ELISA (coating of the ELISA plate with non-transfected CHO-K1 cell proteins instead of r-hu-ECE-1 as coating antigen).

Positive hybridoma cells were subcloned and grown in a serumfree medium (PFHMII, supplemented with 10% (v/v) Opti-mAb; Life Technologies).

All cells were grown in humidified incubators with 5% CO_2 at 37 °C. The monoclonal antibodies were purified on protein A glass beads (Prosep A; Bioprocessing) according to the manufacturer´s protocol.

Immunoblot Analysis

Membrane fractions of CHO-K1 cells (ATCC CCL 61), human ECE-1 transfected CHO-K1 (14) cells and MRC-5 cells (ATCC CCL171; containing

human NEP 24.11) as well as rat/mouse/bovine lung preparations were separated on a 4-20% SDS-polyacrylamid gel under non-reducing conditions, transferred to a nitrocellulose membrane and incubated with the different anti ECE-1 antibodies or anti CD10 mAb (Dianova) as positive control of NEP 24.11. The staining was performed with biotinylated anti-mouse antibody (Sigma) / streptavidin-alkaline phosphatase complex (Boehringer Mannheim) / NBT-BCIP substrate (Boehringer Mannheim)

ECE-1 Neutralization/Immunoprecipitation

Purified r-hu-ECE-1, diluted in 100 mM phosphate buffer pH 7.2, 500 mM NaCl was incubated during 4 h at 4°C with different amounts of mAb´s (up to 10 fold molar surplus).

For the immunoprecipitation protein G sepharose (Sigma) was added during the incubation and removed afterwards by centrifugation.

The enzyme reaction was started by addition of human big-ET-1. After 4 h of incubation at 37°C the reaction was stopped by addition of 0.5% trifluoro-acetic acid and the samples were analyzed by RP-HPLC for the generation of ET-1.

ECE-1 Sandwich ELISA

The ELISA microtiter plates were coated with 5 µg anti-ECE-1 mAb E15/6 /ml 0.1 M $NaHCO_3$ buffer pH 9.2 and left overnight at 4°C. Nonspecific binding sites

were subsequently blocked with 1% BSA in PBS (1 h at 18-25°C). A 2 fold serial dilution of purified ECE-1 starting with 100 ng/ml was incubated for 90 min at 18-25°C. ECE-1 was detected by incubation with 2.5 µg/ml rabbit poly anti-ECE-1 (90 min / 18-25°C) followed by biotinylated anti-rabbit-antibody (1:1000, Sigma; 90 min / 18-25°C) and streptavidin-peroxidase-complex (1:10000, Boehringer Mannheim; 30 min incubation at 18-25°C) / tetramethylbenzidine substrate (Serva). Between each incubation step the plates were washed 3 times with PBS/0.05% Tween 20; all dilutions were made in PBS/0.1% BSA/0.1% Tween 20. The enzymatic reaction was stopped by addition of 2M H_2SO_4 and the absorbance was measured at 450 nm.

To quantify the amount of ECE-1 in samples they were serially diluted and tested in parallel to the ECE-1 standard.

Immunostaining of Cells

CHO-K1 cells and hu-ECE-1 transfected CHO-K1 cells (14) were grown in DMEM containing 10% (v/v) fetal calf serum in 24-well plates until confluency. After fixation with 4% formaldehyde and blocking with 1% BSA in PBS the cells were incubated with the mAb´s (5 µg/ml PBS/0.1% BSA/0.05% Tween 20). Bound antibodies could be detected with biotinylated anti-mouse-antibody (1:10000; Sigma) / streptavidin-alkaline phosphatase complex (1:10000; Boehringer Mannheim) / pNPP substrate (Boehringer Mannheim). The generated nitrophenol in the supernatant was quantified at 405 nm.

Affinity Chromatography

The monoclonal antibody E15/6 was coupled to an ImmunoPure Protein-G-Orientation matrix (Pierce) according to the manufacturer's protocol. Cell culture supernatants containing recombinant human ECE-1 (soluble form) from transfected CHO-K1 cells were purified on this immunoaffinity column; elution of ECE-1 by 0.58% acetic acid, 140 mM NaCl, pH 2.8. ECE-1 containing eluate was neutralized immediately after elution by 0.1 M NaOH.

RESULTS AND DISCUSSION

Characterization of anti-ECE-1 Antibodies

Six cell lines producing anti-ECE-1 monoclonal antibodies have been generated with the characteristics shown in table 1.

The immunoblot (figure 1) under non-reducing conditions results in one strong signal at 250 kDa (ECE-1 dimer) with all mAb's tested. In contrast the blot with the polyclonal rabbit anti-ECE-1 Ab shows also a reaction with several CHO-K1 proteins. Therefore the protein-A purified polyclonal antibody is not specific for ECE-1 and is not suitable for immunoaffinity chromatography, immunohistochemistry etc.

TABLE 1

Characterization of anti-ECE-1 mAb's (*a.* direct ELISA: coating with r-hu-ECE-1; *b.* sandwich ELISA: coating with poly anti-ECE-1)

mAb	antibody subtype	ELISA direct[a] / sandwich[b]		western blot	neutral. of ECE-1 activity	immuno-precipitation of ECE-1
A147/3	IgG_{2b}/κ	+	-	+	-	-
B61/104	IgG_1/κ	+	-	+	-	-
E15/6	IgG_1/κ	+	+	+	-	+
E33/15	IgG_1/κ	+	+	+	-	+
G2/16	IgG_1/κ	+	+	+	-	+
G3/15	IgG_1/κ	+	+	+	-	+

None of the mAb's could neutralize the enzymatic activity of ECE-1 even in 10fold molar surplus. 4 out of 6 antibodies react with native ECE-1 (positive immunoprecipitation) whereas the mAb's A147/3 and B61/104 only recognize ECE-1 under denaturing conditions like western blot analysis or partially in a direct ELISA approach.

None of the six mAb's cross reacts with the metalloprotease NEP 3.4.24.11, which has a significant homology to ECE-1. Only the mAb's B61/104 and A147/3 could recognize ECE-1 of other species than human with a good reactivity. All antibodies are directed against an extracellular epitope of ECE-1 (reaction with an articial soluble form of ECE-1 consisting of the extracellular domain) (table 2).

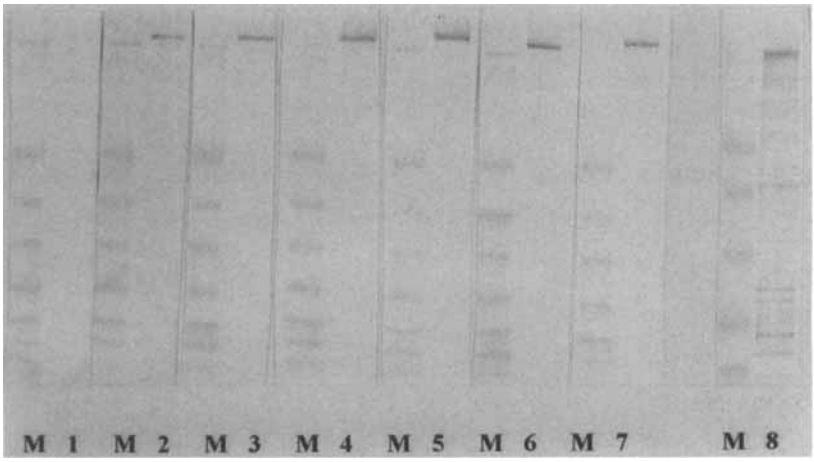

FIGURE 1

Immunoblot analysis of human-ECE-1 transfected CHO-K1 cells:
proteins separated by SDS-PAGE (4-20%) under non-reducing conditions;
staining with anti-ECE-1 mAb´s / biotinylated anti-mouse antibody /
streptavidin-alkaline phosphatase complex / NBT-BCIP substrate

lane **1**	control	lane **5**	mAb E33/15
lane **2**	mAb A147/3	lane **6**	mAb G2/16
lane **3**	mAb B61/104	lane **7**	mAb G3/15
lane **4**	mAb E15/6	lane **8**	poly anti-ECE-1

M marker (250, 98, 64, 50, 36, 30, 16 kDa)

The 4 antibodies recognizing native ECE-1 are directed against an overlapping epitope: no combination of 2 mAb´s bind to soluble monomeric ECE-1 (table 3). In contrast the dimeric ECE-1 (containing 2 of each epitopes) reacts in a sandwich ELISA approach. This immunodominant epitope of native ECE-1 is specific for the human enzyme (no reactivity was observed with rat/mouse/bovine ECE-1).

ECE-1 Sandwich-ELISA

Because each epitope is present twice in the dimeric ECE-1 all pairs of 2 mAb´s

TABLE 2

Specificity of anti-ECE-1 mAb´s tested by reactivity in western blot analysis (*a.* neutral endopeptidase 3.4.24.11; *b.* soluble monomeric ECE-1, consisting of the extracellular domain)

	NEP[a)]	**ECE-1**				**s-ECE-1**[b)]
	human	mouse	bovine	rat	human	human
mAb						
A147/3	-	+	+	+	+	+
B61/104	-	+	+	+	+	+
E15/6	-	-	-	-	+	+
E33/15	-	-	-	-	+	+
G2/16	-	-	-	-	+	+
G3/15	-	-	-	-	+	+

TABLE 3

Reactivity of different mAb-combinations in an ECE-1 sandwich-ELISA: coating of ELISA plates with mAb´s, incubation with **soluble monomeric ECE-1 / dimeric ECE-1**, detection with biotinylated mAb´s (modification of the antibodies by sulfo-NHS-biotin according to a Pierce protocol), streptavidin peroxidase complex and tetramethylbenzidine substrate

biotinylated mAb	coating mAb			
	E15/6	**E33/15**	**G2/16**	**G3/15**
E15/6	- / +	- / +	- / +	- / +
E33/15	- / +	- / +	- / +	- / +
G2/16	- / +	- / +	- / +	- / +
G3/15	- / +	- / +	- / +	- / +

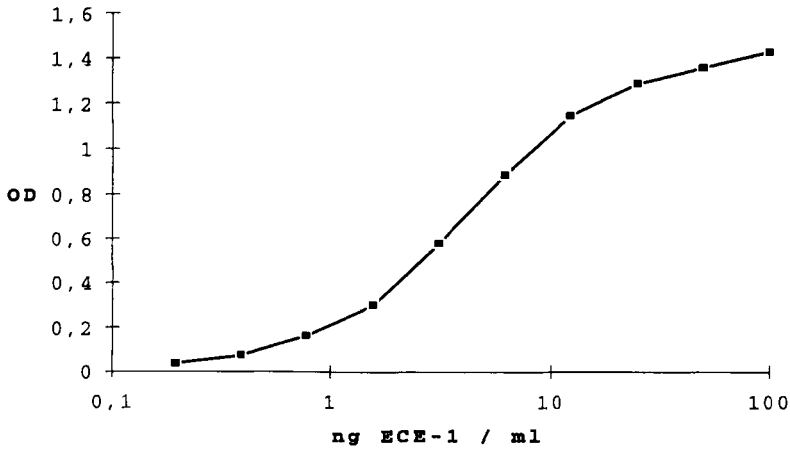

FIGURE 2

Sandwich ELISA to detect human ECE-1; ECE-1 concentration vs. OD; coating of the ELISA plate with mAb E15/6; serial dilution of soluble, monomeric r-hu-ECE-1; detection with poly anti ECE-1, biotinylated anti-rabbit antibody; streptavidin peroxidase complex, tetramethylbenzidine substrate

are suitable for a sandwich ELISA approach. Nothing but the combination of a mAb and the polyclonal Ab is appropriate for the set-up of an ECE-1 sandwich-ELISA to detect monomeric ECE-1 (figure 2).

By this type of assay (combination of mAb E15/6 and poly anti-ECE-1) we could quantify dimeric ECE-1 as well as a soluble monomeric form of ECE-1 with a detection limit of about 0.5 ng/ml. There is no cross-reactivity with other proteases, even with the closely related NEP 24.11 (cross-reactivity < 0,1%). The validation was performed by studying the repeatability (intra-assay variation) and reproducibility (inter-assay variation). All data were obtained with standard curves

TABLE 4

Validation of ECE-1 sandwich ELISA (intra-assay: n=8; inter-assay: n=5)

ECE-1 [ng/ml]	ECE-1 detected [ng/ml]	accuracy [%]	CV intra-assay [%]	CV inter-assay [%]
25	23.470	93.9	15.0	12.4
12.5	12.501	100.0	6.7	7.4
6.25	6.338	101.4	4.4	3.5
3.125	3.102	99.3	4.7	8.2
1.562	1.553	99.4	3.2	8.5
0.781	0.794	101.6	3.7	9.9
0.391	0.404	103.2	13.6	14.6

consisting of PBS/0.1%BSA/0.1% Tween 20 spiked with recombinant human ECE-1 (table 4).

With this ELISA attempts were made to detect ECE-1 in human plasma. In preliminary studies it was possible to detect ECE-1 immuno-reactivity in the plasma of patients; the concentration varies between 0 and greater 500 ng/ml (data not shown). At present possible correlations to a specific pathology and the heterogeneity of this immunoreactive ECE-1 are investigated. Preceding results detect ECE-1 activity, quantified by the cleavage of big-ET-1 followed by a ET-1 sandwich ELISA, in the human serum lipoprotein fraction (22).

In analogy to NEP 24.11 and several other enzymes it might be possible that a soluble form of ECE-1 exists. The physiological relevance and enzymatic activity

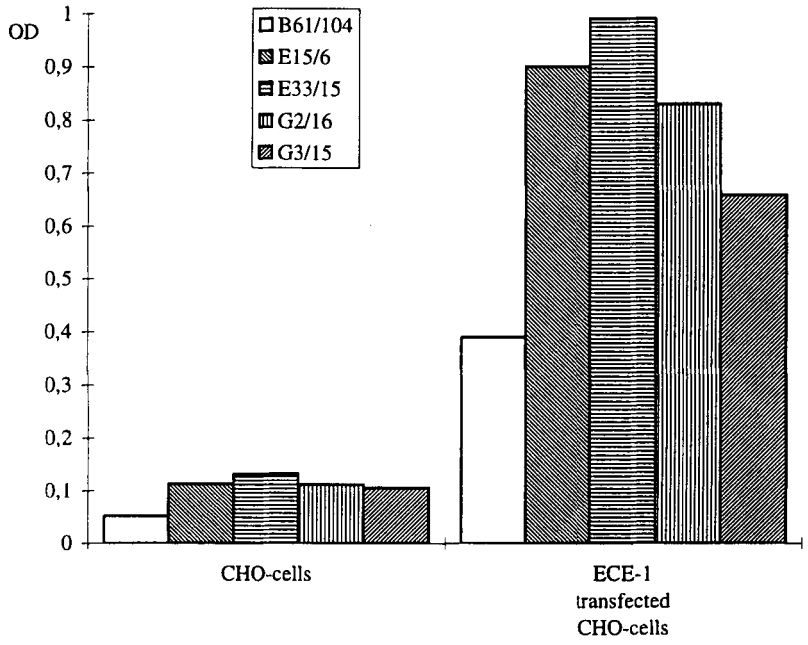

FIGURE 3

Immunostaining of non-transfected and r-hu-ECE-1 transfected CHO-K1 cells with anti-ECE-1 mAb's (mAb A147/3 n.d.); incubation with biotinylated anti-mouse antibody; streptavidin alkaline phosphatase complex; pNPP substrate; the OD of nitrophenol in the supernatant was detected at 405 nm

of these fragments have to be shown (the artificial soluble form of ECE-1 consisting of the extracellular part has enzymatic activity). The ECE-1 immunoreactivity could result from unspecific proteolysis or could be involved in the regulation of the ET-1 release.

Immunocytochemistry

The described anti-ECE-1 mAb's are suitable for immunocytochemical staining

of human ECE-1 in the cell membrane of transfected CHO-K1 cells after non-permeabilized formaldehyde fixation (figure 3).

With the described mAb´s it is possible to detect ECE-1 in several tissues by immunohistochemistry (data not shown).

Recent results with different anti-ECE-1 mAb´s have localized ECE-1 in neo-intimal smooth muscle cells in rat carotid arteries after balloon injury and in smooth muscle cells and macrophages in human coronary atherosclerotic lesions (11).

Affinity Purification of ECE-1

Affinity purification of recombinant human ECE-1 was possible by coupling of the mAb´s to an ImmunoPure Protein G orientation matrix. As expected only the 4 mAb´s recognizing ECE-1 in its native form are successful in affinity chromatography. ECE-1 bound to the matrix could be eluted under acidic conditions. Fast neutralization was necessary to prevent loss of enzymatic activity. Starting from serumfree cell culture supernatants ECE-1 could be purified to about 90% (estimation by silver staining SDS-PAGE) by single-step affinity chromatography. The soluble form of ECE-1 produced in transfected CHO-K1 cells results in a single signal at about 125 kDa (figure 4).

This purified ECE-1 will be used to further characterize the biochemical properties and to identify ECE-1 inhibitors which might be useful in the therapy of different pathological situations. A 3-D structure of the enzyme will facilitate the

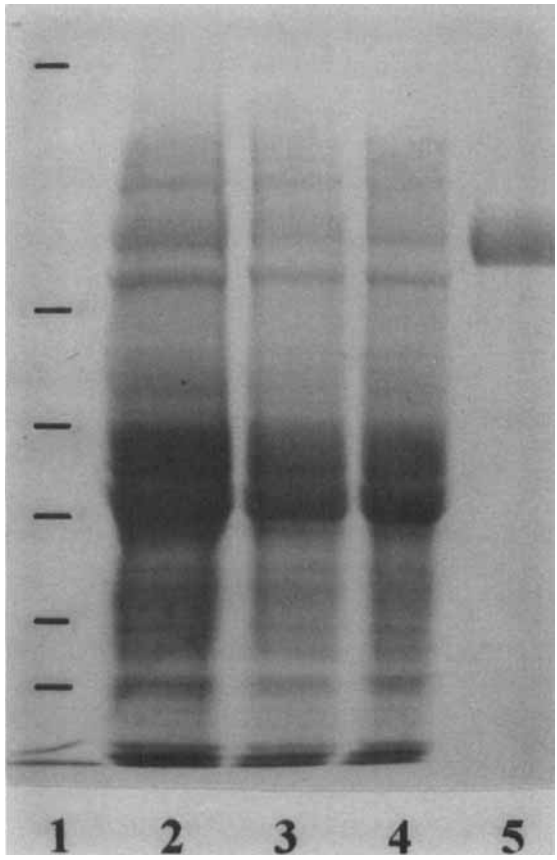

FIGURE 4

SDS-PAGE analysis of fractions obtained by affinity chromatography of soluble monomeric ECE-1 on a mAb E15/6 immunomatrix (4-20% gradient polyacrylamide gel under non-reducing conditions, stained with Coomassie brilliant blue)

lane **1** marker (250, 98, 64, 50, 36, 30 kDa)
lane **2** cell culture supernatant
lane **3** unbound fraction
lane **4** wash
lane **5** purified ECE-1

rational drug design of ECE-1 inhibitors. The immunoaffinity chromatography is one essential step in the purification of ECE-1 for crystallization experiments.

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