Isolation and characterization of a trypsin-like serine proteinase from the membranes of Walker 256 carcino-sarcoma cells

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A serine proteinase was isolated from Walker-256 carcino-sarcoma plasma-membrane-enriched preparations by affinity chromatography employing soybean trypsin inhibitor as the ligand. This enzyme was termed 'memsin' owing to its membrane location and trypsin-like substrate specificity. Analysis of this preparation by steric-exclusion high-pressure liquid chromatography (h.p.l.c.) resulted in a single peak of enzyme activity. Calculations of the rates of inactivation of memsin by peptidyl-chloromethanes and comparison with rate constants obtained with other serine proteinases indicated that memsin closely resembled trypsin and acrosin. Digestion of oxidized ribonuclease by memsin and analysis of the resulting peptides by h.p.l.c. yielded a chromatogram that was very similar to one generated by a tryptic digest of oxidized ribonuclease. This enzyme could possibly play a role in tumour-cell invasion.

In the present study we describe the isolation of a serine proteinase, 'memsin', from a Triton X-100 extract of the Walker 256 plasma membrane by affinity chromatography. The relationship of this proteinase to other well-characterized serine proteinases was investigated by the determination of active-site specificity employing synthetic peptidyl-chloromethanes. This, we believe, is the first report on the isolation of a membrane-bound trypsin-like enzyme.

Materials and methods

Serially transplanted Walker 256 carcino-sarcoma cells were obtained in single-cell suspension from the ascites that develops 7 days after an intraperitoneal implantation of $1 \times 10^6$ tumour cells into male Wistar rats. Tumour cells with less than 2% contamination by other cell types, as demonstrated by Zucker & Lysik (1977), were isolated on Ficoll/Hypaque gradients (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) and washed repeatedly with Hanks balanced salt solution (Flow Laboratories, McLean, VA, U.S.A.). Tumour-cell membrane-enriched fractions were prepared as previously described (DiStefano et al., 1982). Briefly, the cells were lysed by mixing in a hypo-osmotic NaHCO$_3$ solution and then subjected to differential centrifugation. The material that

The role of membrane-bound proteinases in tumour cells is not entirely clear. Hatcher et al. (1976) have shown an increase in the amount of proteinases on the surface of transformed mouse epithelial cells, and correlated this with a decrease in cell doubling time. Quigley et al. (1980) have found a plasminogen activator associated with the membrane fraction of virus-transformed chick-embryo fibroblasts.

Membrane-bound serine proteinases have been previously described on the surface of Walker 256 carcino-sarcoma cells by this laboratory (DiStefano et al., 1982). Triton X-100 extracts of a membrane-enriched fraction were shown to be enzymically active against the synthetic substrate Tos-Arg-OMe. This activity could be inhibited by the broad-spectrum serine-proteinase inhibitor di-isopropyl phosphofluoridate (iPr$_2$-P-F), and Tos-Lys-CH$_2$Cl, an inhibitor of trypsin-like enzymes.

Abbreviations used: Tos, tosyl; OMe, methyl ester; iPr$_2$-P-F, di-isopropyl phosphofluoridate; CH$_2$Cl, chloromethane; SBTI, soybean trypsin inhibitor; CH-Sepharose, 6-aminoexanoc acid-Sepharose; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride; SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography; SBzl-Z-lys, thio-phenylalanine-carbonyl-lysine; Pipes, 1,4-piperazinediethanesulphonic acid.

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Affinity chromatography

Soya-bean trypsin inhibitor (SBTI; Sigma Chemical Co., St. Louis, MO, U.S.A.) was coupled to CH-Sepharose 4B (Pharmacia) via a carbodi-imide-mediated condensation reaction. The coupling-reaction mixture consisted of 10 ml of swelled CH-Sepharose 4B in distilled water titrated to pH 4.5, and 60 mg of SBTI in distilled water to which 5 ml of a 20 mg/ml solution of EDC was added dropwise with constant mixing. Coupling was effected at room temperature with end-over-end mixing for 1 h with constant pH adjustments. The coupling reaction was allowed to continue for 16 h at 4°C. After washing out any unbound ligand, groups that had not reacted were subsequently blocked by a second carbodi-imide reaction with glucosamine. After washing out excess ligand, the gel was stored at 4°C in 0.1 M-Tris/HCl, pH 8.5, containing 0.1% Triton X-100, 0.5 M-NaCl and 0.02% NaN₃.

The SBTI–CH-Sepharose 4B gel was poured into a column (1.6 cm × 5 cm) and washed extensively with buffer consisting of 0.1 M-Tris/HCl (pH 8.5)/0.1% Triton X-100, before sample application. A 10 ml portion of the diluted Triton X-100-solubilized membrane preparation was applied to the column with a flow rate of 20 ml/h. The column was washed with 10–20 column volumes of starting buffer. Elution was accomplished utilizing a stepwise gradient consisting of starting buffer containing 0.1, 0.3, 0.5 and 1.0 M-NaCl. Elution was completed with a 0.1 M-sodium acetate buffer, pH 4.0, containing 0.5 M-NaCl. Each of the acetate fractions were titrated to pH 7.4 with 0.1 M-NaOH immediately upon collection.

Electrophoresis in Triton-containing acrylamide gels

The affinity-purified enzyme (memsin) was subjected to electrophoresis in 4%-acrylamide gels containing 0.1% Triton X-100. Electrophoresis was carried out in a Bio-Rad polyacrylamide-disc-gel-electrophoresis system (Bio-Rad, Rockville Center, NY, U.S.A.), with 5 mm × 75 mm gel tubes being used. The bottom electrode chamber contained 0.1 M-Tris/glycine buffer, pH 8.3, whereas the top electrode chamber contained the same buffer with the addition of 0.1% Triton X-100. The gels were run at 4°C for 2 h at 2 mA/gel. At the completion of the run, duplicate gels were stained for non-specific esterase activity with α-naphthyl acetate (DeWald et al., 1974) and for protein with Coomassie Blue (Chrambach et al., 1967).

Electrophoresis in SDS-containing acrylamide gels

Electrophoresis in gels containing 0.1% SDS was performed by the method of Segrest & Jackson (1972). Memsin was run in 5 mm × 75 mm gels containing 7.5, 10, 12.5 and 15% acrylamide. The electrophoresis run was terminated when the tracking dye reached within 2 mm of the bottom of the tubes and the protein bands stained with Coomassie Blue. To estimate the molecular size of memsin, SDS/polyacrylamide-gel calibration proteins (Pharmacia) were run concurrently at each acrylamide concentration.

H.p.l.c.

In an attempt to purify the enzyme further, h.p.l.c. was performed on a Laboratory Data Control h.p.l.c. system employing a 0.8 cm × 30 cm TSK 2000 SW column (Toyo Soda Manufacturing Co., Tokyo, Japan). Isocratic elution was performed with a 0.1 M-Na₂SO₄/0.2 M-NaH₂PO₄ buffer, pH 6.8, at a flow rate of 0.17 ml/min. The effluent was monitored at 210 nm and resulting peaks were collected on ice for enzymic analysis. To estimate the molecular size of the resulting protein peaks the column was calibrated with proteins of known molecular size. These standards included: albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000) and anhydrotrypsin (mol.wt. 22000).

Enzyme assays

The esterolytic activities of the various fractions were measured by using the synthetic substrate Tos-Arg-OMe (Kettner & Shaw, 1978). Portions of the enzyme were incubated in the presence of inhibitors at 25°C in 50 mM-Pipes buffer, pH 7.0, containing 0.2 M-NaCl. At selected time intervals a sample was removed and assayed against the substrate SBzl-Z-Lys. Where possible, rates of inactivation were determined at inhibitor concentrations that resulted in a half-time of inactivation of 15–30 min. Apparent pseudo-first-
order rates of inactivation were then obtained from semi-logarithmic plots of enzyme activity versus time.

Proteolytic action of memsin on oxidized ribonuclease

To determine the proteolytic specificity of memsin, oxidized ribonuclease (Schwarz–Mann, Orangeburg, NY, U.S.A.) was digested by the enzyme preparations and the resulting peptides analysed by h.p.l.c. The reaction mixture consisted of 150 μl of a 12 mg/ml solution of oxidized ribonuclease in 0.1 M N-methylmorpholine acetate, pH 8.0, to which was added 50 μl of the enzyme preparation. For comparison, a solution of trypsin at 33 μg/ml in 10 mM HCl was employed. This was equivalent in esterase units to memsin as assayed with SBzl-Z-Lys. The process of digestion was monitored by h.p.l.c. on a RP18 Liscorsorb column (0.46 cm × 30 cm; EM Reagents, Houston, TX, U.S.A.), with 20 μl of the reaction mixture. Elution was accomplished with a 20 mM ammonium acetate buffer, pH 5.6, employing a gradient of acetonitrile from 2 to 50% developed for 20 min at a flow rate of 2 ml/min. The eluate was monitored at 230 nm.

Results

Affinity chromatography of the Triton X-100-solubilized membranes on SBTI–CH-Sepharose 4B yielded several fractions with activity towards Tos-Arg-OMe. The fractions that were found to be highest in specific activity were those derived from the acetate-buffer-elution step. The specific activities of each of the resulting fractions from a typical run are given in Table 1. Specific activities are expressed in units (μmol of Tos-Arg-OMe hydrolysed/min) per mg of protein. As is evident, the acetate fraction contains the highest specific activity and was therefore chosen for continued study. The specific activities of the acetate-buffer-derived fractions ranged from 16.92 to 75.40 Tos-Arg-OMe units/mg of protein, with a mean of 48.40.

Memsin was subsequently subjected to analysis by steric-exclusion chromatography on h.p.l.c. Fig. 1 is a chromatogram of memsin eluted through a TSK 2000 SW exclusion column. Two peaks were resolved by this method, the first of which was shown to contain all the enzyme activity that was applied to the column. The second peak, which appeared at the total volume of the column, consisted of residual non-protein-bound detergent. The enzyme-containing protein peak eluted at a position corresponding to a mol wt of 20 100.

Electrophoresis in Triton X-100-containing polyacrylamide gels

Memsin was subjected to electrophoresis in 4%-acrylamide gels containing 0.1% Triton X-100.

Table 1. Activities towards Tos-Arg-OMe of the fractions derived from affinity chromatography of the Triton X-100-solubilized Walker 256 plasma membranes on SBTI–CH-Sepharose 4B

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (μmol of Tos-Arg-OMe hydrolysed/min)/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized membranes</td>
<td>0.019</td>
</tr>
<tr>
<td>Unbound</td>
<td>0.500</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
</tr>
<tr>
<td>0.1 M</td>
<td>0.548</td>
</tr>
<tr>
<td>0.3 M</td>
<td>0.868</td>
</tr>
<tr>
<td>0.5 M</td>
<td>0.850</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.122</td>
</tr>
<tr>
<td>Acetate buffer</td>
<td>52.810</td>
</tr>
</tbody>
</table>

Fig. 1. H.p.l.c. of memsin

The affinity-purified enzyme was analysed by h.p.l.c. a TSK 2000 SW exclusion column (0.8 cm × 30 cm) being used. Isocratic elution was accomplished with a 0.1 M Na₂SO₄/0.2 M NaH₂PO₄ buffer, pH 6.8, at a flow rate of 0.17 ml/min. The effluent was monitored at 210 nm. Column calibration was performed with the protein standards albumin (Alb), ovalbumin (Ovalb) and anhydrotrypsin (Antryp).

After the termination of the run the gels were stained for non-specific esterase activity with a-naphthyl acetate. The affinity-purified enzyme was shown to resolve into four bands, which stained for esterase activity (results not shown); a duplicate gel stained for protein with Coomassie Blue yielded the identical four bands (results not shown).

SDS/polyacrylamide-gel electrophoresis

In an attempt to estimate the molecular size of the components present in the affinity-purified enzyme preparation, electrophoresis was performed in several SDS-containing gels of increasing acrylamide concentration. By employing the procedure of Segrest & Jackson (1972), an asymptotic minimum
molecular size was estimated. The gels revealed the presence of four protein bands (results not shown). The major band was estimated to have a molecular size of 32,500 Da. The remaining three bands were shown to possess molecular sizes, ranging from top to bottom, of 59,000, 43,000 and 23,000 Da.

**Rates of inactivation of memsin by peptidyl-chloromethanes**

The kinetic rate constants for the inactivation of memsin by peptidyl-chloromethanes are shown in Table 2. As is evident, peptidyl-arginylchloromethanes demonstrated significant inhibitory capabilities against memsin. When homoarginine was substituted for arginine in the P₁ position, the resulting chloromethane was approx. 3000-fold less inhibitory than the identical peptidyl-arginyl-chloromethane. Proline in the P₂ position was shown to confer the greatest specificity for memsin, closely followed by glycine. Phenylalanine at the P₃ position conferred the least amount of specificity of the arginyl-chloromethanes tested. Lysine at the P₁ position was also shown to be effective for the inhibition of memsin.

**Comparison of the rates of inactivation of memsin by peptidyl-chloromethanes**

Table 3 outlines the relationship of memsin to other well-characterized serine proteinases, by comparing the rates of inactivation of these enzymes by chloromethanes. As is apparent from the present study, the substrate specificity of memsin resembles least those of thrombin, Factor Xa and plasmin. For example, the specificity of Pro-Gly-Arg-CH₂Cl for memsin is approx. 100-fold that for thrombin, 60-fold that for Factor Xa and 1000-fold that for plasmin. The substrate specificity of memsin more closely resembles those for acrosin and trypsin. The specificity of Pro-Gly-Arg-CH₂Cl for memsin was approx. 2-fold that of acrosin and trypsin. When glutamine was substituted for proline at the P₂ position, memsin was 2.5 times less sensitive to inhibition than was acrosin, and 4.5 times less susceptible than was trypsin. These data indicate that memsin possesses an active site that is similar to that of both trypsin and acrosin, but in turn is distinct.

**Digestion of oxidized ribonuclease by trypsin and memsin**

The digestion of oxidized ribonuclease by trypsin and memsin was allowed to progress to completion, which was seen to occur in 5.4 h. Fig. 2(a) depicts an h.p.l.c. chromatogram of oxidized ribonuclease before incubation with enzymes. The chromatograms in Figs. 2(b) and 2(c) describe the peptides which result from the complete digestion of oxidized ribonuclease by trypsin and memsin respectively. As is apparent from these chromatograms, both species of enzymes yielded similar profiles from complete digestion of the oxidized ribonuclease.

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**Table 2. Rates of inactivation of memsin by chloromethanes**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc. (m)</th>
<th>tᵢ* (min)</th>
<th>10⁻⁴ × kᵧₓₓₓ · /[II]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Gly-Arg-CH₂Cl</td>
<td>4 × 10⁻⁸</td>
<td>14.00</td>
<td>124</td>
</tr>
<tr>
<td>Glu-Gly-Arg-CH₂Cl</td>
<td>2 × 10⁻⁸</td>
<td>24.65</td>
<td>140</td>
</tr>
<tr>
<td>Pro-Phe-Arg-CH₂Cl</td>
<td>5 × 10⁻⁷</td>
<td>15.99</td>
<td>8.7</td>
</tr>
<tr>
<td>Val-Pro-Arg-CH₂Cl</td>
<td>2.5 × 10⁻⁷</td>
<td>15.32</td>
<td>180</td>
</tr>
<tr>
<td>Glu-Gly-Harg-CH₂Cl</td>
<td>1 × 10⁻⁴</td>
<td>19.09</td>
<td>0.036</td>
</tr>
<tr>
<td>Phe-Phe-Arg-CH₂Cl</td>
<td>2.5 × 10⁻⁶</td>
<td>10.55</td>
<td>26.3</td>
</tr>
<tr>
<td>Pro-Phe-Pro-Arg-CH₂Cl</td>
<td>5 × 10⁻¹⁰</td>
<td>28.29</td>
<td>4900</td>
</tr>
<tr>
<td>Ala-Phe-Lys-CH₂Cl</td>
<td>2 × 10⁻⁶</td>
<td>6.9</td>
<td>5</td>
</tr>
</tbody>
</table>

* Half-time for the pseudo-first-order rate of inactivation.
† Estimate of the second-order rate constant.

**Table 3. Rates of inactivation of serine proteinases by chloromethanes**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Memsin</th>
<th>Thrombin</th>
<th>Factor Xa²†</th>
<th>Plasmin</th>
<th>Acrosin³</th>
<th>Trypsin³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-Gly-Arg-CH₂Cl⁴⁺</td>
<td>124</td>
<td>1.2⁴</td>
<td>2.0</td>
<td>0.11⁴</td>
<td>67</td>
<td>70</td>
</tr>
<tr>
<td>Glu-Gly-Arg-CH₂Cl⁴⁺</td>
<td>140</td>
<td>2.2⁴</td>
<td>14</td>
<td>1⁴</td>
<td>370</td>
<td>620</td>
</tr>
<tr>
<td>Pro-Phe-Arg-CH₂Cl⁴⁺</td>
<td>8.7</td>
<td>0.1¹</td>
<td>1.3</td>
<td>1.9¹</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>Val-Pro-Arg-CH₂Cl⁴⁺</td>
<td>180</td>
<td>54⁵</td>
<td>0.028</td>
<td>0.3²</td>
<td>160</td>
<td>430</td>
</tr>
<tr>
<td>Glu-Gly-Harg-CH₂Cl⁴⁺</td>
<td>0.036</td>
<td>N.D.</td>
<td>4.7</td>
<td>2.6²</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Phe-Phe-Arg-CH₂Cl⁴⁺</td>
<td>26.3</td>
<td>N.D.</td>
<td>N.D.</td>
<td>21¹</td>
<td>0.88</td>
<td>3.6</td>
</tr>
<tr>
<td>Ala-Phe-Lys-CH₂Cl⁴⁺</td>
<td>5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

| 10⁻⁴ × kᵧₓₓₓ · /[II] (m⁻¹·min⁻¹) |
|------------------|---|---|---|---|---|---|
| 0.12 |

² Indicates the reference for the synthesis of the indicated compound; † indicates the reference from which the kᵧₓₓₓ · /[II] values were obtained. References: ¹Kettner & Shaw (1978); ²Kettner & Shaw (1981); ³Kettner et al. (1978); ⁴Kettner & Shaw (1979); ⁵Kettner & Shaw (1977); ⁶Kettner et al. (1980). Abbreviation used: N.D., not determined.
Discussion

In the past, the purification of membrane-bound enzymes has proved to be difficult [see Juliano (1978) for a review]. The use of conventional column-chromatographic techniques, although still possible, is cumbersome, owing to the inclusion of high concentrations of detergents that are necessary to maintain the membrane-derived proteins in soluble form. The use of various lectins coupled to an insoluble matrix has gained wide acceptance for the identification of membrane lectin receptors (Gurd & Mahler, 1974; Juliano, 1978; Nachbar et al., 1976; Rakonczay et al., 1981). However, this technique alone seldom leads to a highly purified preparation (Juliano, 1978). The use of reversible enzyme inhibitors covalently coupled to agarose is an effective tool for the isolation of enzymes. This technique does have disadvantages, in that the binding of enzymes to immobilized inhibitors can occasionally be so strong that they are essentially irreversible (Saklatvala & Barrett, 1980).

In the present investigation we have employed SBTI covalently coupled to Sepharose 4B through a six-carbon spacer group. This method has proven to be effective for the isolation of proteinases associated with the membrane of Walker 256 cancer cells. The serine proteinase isolated, memsin, showed a very high affinity for the SBTI–CH-Sepharose 4B column, since it can not be dissociated in the presence of 1 M-NaCl. Attempts to purify this enzyme with SBTI coupled directly to Sepharose 4B have not been successful, indicating some degree of steric interference on the enzyme–inhibitor complex.

To ascertain information as to the specificity of the active site of memsin, peptidyl-chloromethanes were employed. By calculating the pseudo-first-order kinetics of the rates of inactivation, an inhibitor profile can be obtained and compared with those previously obtained for other serine proteinases. Only chloromethanes were used in the present study, since all preliminary data pointed to the presence of a serine proteinase. As Table 3 shows, the profiles of memsin clearly resemble those of trypsin and, to a lesser extent, acrosin. These enzymes are in general very sensitive to peptidyl-chloromethanes, but not very sensitive to minor sequence variations in the peptidyl moiety. This is in contrast with the more specialized trypsin-like enzymes such as thrombin and Factor Xa. Memsin’s resemblance to trypsin is also suggested by its desorption from a trypsin-inhibitor column.

In addition to these results, the digestion of oxidized ribonuclease by memsin yields a set of peptides that are closely analogous to those of a tryptic digest. Acrosin has also been reported by Polakoski & McRorie (1973) to yield a complete digest of this type.

The presence of a trypsin-like proteinase on the surface of the Walker 256 carcino-sarcoma cell is consistent with the tumour cell’s ability to invade...
surrounding normal tissue. The increased proteolytic capability of tumour cells is well documented (Bosmann et al., 1973; Liotta et al., 1980; Unkeless et al., 1973). On the basis of our previous studies utilizing an experimental model of cancer invasion (Zucker & Lysik, 1977), we postulate that this enzyme most probably plays a role in the invasiveness of the Walker cancer. The role of cell-membrane-bound serine proteinase in the basic pathophysiology of cancer invasion remains to be more fully explained. Additional work is required to isolate the remaining proteinases which appear to be present on the membrane of the Walker 256 carcino-sarcoma cell.

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