PURIFICATION AND COMPARISON OF SEVERAL CATALYTIC PARAMETERS OF THE \( \gamma \)-GLUTAMYLTRANSPEPTIDASE OF RAT MAMMARY ADENOCARCINOMA (13762) AND OF NORMAL RAT MAMMARY GLAND

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Summary

A method for the purification of a membrane-bound glycoprotein, \( \gamma \)-glutamyltranspeptidase (\( \gamma \)-glutamyl-peptide:amino-acid \( \gamma \)-glutamyltransferase, EC 2.3.2.2), from a transplantable rat mammary tumor (13762 MT) is described. The properties of the tumor enzyme were compared with those of \( \gamma \)-glutamyltranspeptidase similarly isolated from mammary tissue of non-pregnant multiparous rats. Evidence has been presented elsewhere that the mammary and tumor enzymes exist as groups of species differing in isoelectric point and that the tumor enzyme contains more of those species with lower isoelectric points. In this study the normal and tumor enzyme preparations are found to be identical or very similar in regards to the effect of papain on molecular size, the ratios of the enzymatic activities as measured with various amino acids, the \( K_m \) for \( \gamma \)-glutamyl-p-nitroanilide, and the \( K_i \) for inhibition by glutathione. Neuraminidase treatment had no effect on these catalytic properties. The properties observed were generally similar to those previously reported for highly purified rat kidney preparations.

Introduction

\( \gamma \)-Glutamyltranspeptidase (\( \gamma \)-glutamyl)-peptide:amino-acid \( \gamma \)-glutamyltransferase, EC 2.3.2.2) activity has been suggested to be involved with amino acid
transport [1] and with glutathione metabolism [2,3], processes which have
been reported to undergo changes accompanying malignant transformation.
The activity of this membrane-bound glycoprotein has been associated with
plasma membrane functions because of its localization in a plasma membrane
fraction from kidney [4] and its proposed role in the γ-glutamyl cycle for
amino acid transport [1]. Isolation of this enzyme from normal and malignant
breast tissue and comparisons of the properties of the preparations might there-
fore provide information concerning changes in a functionally important
individual membrane component accompanying transformation.

Several forms of the kidney enzyme, differing in isoelectric point as a reflec-
tion of varying sialic acid content, have been reported [5]. Testicular, seminal
vesicular, and epididymal enzymes have also been reported [6–8] to exist in
isoforms of varying isoelectric point. It has been noted [5] that the relative
proportion of these species may differ in the various rat tissues. We have previ-
ously reported [9] the presence of several such species of the enzyme in rat
mammary tissue and have shown their isoelectric focusing profile to be
markedly different from that of the species observed in rat mammary adenocar-
cinoma (13762). In purifying the enzyme from these sources, published
methods for publication of the kidney enzyme were unsuccessful in our hands.
We now report the method of purification of the enzyme from these two
sources and provide evidence that the catalytic properties of the two prepara-
tions of the two preparations that we have analyzed are similar and are not
distinctly different from those reported for the kidney enzyme [10].

Experimental and Results

Purification of γ-glutamyltranspeptidase from mammary and tumor tissue

All procedures were carried out at 0—4°C except where noted. Glutaral-
dehyde cross-linked concanavalin A [11] was prepared from purified [12] con-
canavalin A. Assay methods are listed in the tables and figures.

Frozen homogenates, prepared from the tumor or normal mammary tissue as
previously described [9], were thawed by gentle shaking in a 37°C water bath.
They were then filtered through four layers of cheesecloth. The residue was
rinsed with homogenizing buffer. This procedure resulted in removal of much
of the fat of the mammary gland homogenate without loss of activity.

The filtered homogenate was centrifuged at 100 000 × g for 60 min. The
pellet was resuspended in 1/4 or 1/2 of the original volume for normal or
tumor tissue, respectively, in 0.01 M phosphate buffer (pH 7.5), 0.1 M KCl,
and 0.2% Triton X-100 (buffer A) using a motor-driven teflon pestle. The pro-
tein concentrations were adjusted to approximately 2 mg/ml or 8 mg/ml for
normal and tumor tissue, respectively. For tumor tissue the recovery at this
step is almost quantitative, with less than 8% of the activity remaining in the
100 000 × g supernatant. However, for mammary tissue, about 25% of the
activity remained in the floating fatty layer above the supernatant and was
discarded.

The cloudy Triton suspension was stirred on ice and 50% polyethylene
glycol 6000 (J.T. Baker Chemical Co.) was added dropwise until a final concen-
tration of 5% was achieved. Stirring was continued for 10 min and the mixture
TABLE I
PURIFICATION OF \( \gamma \)-GLUTAMYLTRANSPEPTIDASE FROM 13762 MT MAMMARY ADENOCARCINOMA
Protein was estimated by the method of Lowry et al. [14]. Total activity is expressed as \( \mu \text{mol} \) \( \rho \)-nitroanilide/min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity</th>
<th>Spec. act. (%)</th>
<th>Yield (%)</th>
<th>Relative Spec. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>160</td>
<td>1900</td>
<td>120</td>
<td>0.06</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Triton suspension</td>
<td>85</td>
<td>635</td>
<td>150</td>
<td>0.23</td>
<td>130</td>
<td>3.8</td>
</tr>
<tr>
<td>5% polyethylene glycol supernatant</td>
<td>87</td>
<td>83.5</td>
<td>115</td>
<td>1.35</td>
<td>100</td>
<td>23</td>
</tr>
<tr>
<td>Concanavalin A eluates</td>
<td>3.2</td>
<td>12</td>
<td>80</td>
<td>6.9</td>
<td>79</td>
<td>114</td>
</tr>
<tr>
<td>Papain treatment and G-100 chromatography</td>
<td>2.0</td>
<td>&lt;5 ( \mu \text{g/ml} ) *</td>
<td>65</td>
<td>( &gt;1320 )</td>
<td>56</td>
<td>( &gt;22000 )</td>
</tr>
</tbody>
</table>

* Because of such small amounts of material, protein in this fraction was estimated by the more sensitive fluorescamine method [15].

was then centrifuged at 15 000 \( \times g \) for 10 min. The resultant clear supernatant was stirred with glutaraldehyde cross-linked concanavalin A in a ratio of 5 ml 50% polyethylene glycol supernatant to 0.25 g wet weight for 1 h. This mixture was then centrifuged for 10 min at 3000 \( \times g \) and the supernatant discarded. For both normal and tumor enzyme preparations, 90–95% of the enzyme activity was adsorbed under these conditions. The remaining activity could not be adsorbed with further treatment. Elution from the cross-linked concanavalin A was achieved by stirring for 8 h three separate times in 5 ml of buffer A with the addition of 20 mg/ml \( \alpha \)-methylglucoside. Each eluate was collected by centrifuging at 3000 \( \times g \) for 10 min. The eluates were then combined and centrifuged at 30 000 \( \times g \) for 20 min, and dialyzed against 0.01 M phosphate buffer (pH 7.5) containing Bio Beads SM 2 (Bio-Rad) to remove

TABLE II
PURIFICATION OF \( \gamma \)-GLUTAMYLTRANSPEPTIDASE FROM MAMMARY TISSUE OF NON-PREGNANT MULTIPAROUS RATS
Protein was estimated by the method of Lowry et al. [14]. Total activity is expressed as \( \mu \text{mol} \) \( \rho \)-nitroanilide/min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity</th>
<th>Spec. act.</th>
<th>Yield (%)</th>
<th>Relative spec. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>114</td>
<td>1115</td>
<td>11.4</td>
<td>0.01</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Triton suspension</td>
<td>25</td>
<td>45</td>
<td>8.8</td>
<td>0.19</td>
<td>77</td>
<td>19</td>
</tr>
<tr>
<td>5% polyethylene glycol supernatant</td>
<td>25</td>
<td>6.2</td>
<td>8.0</td>
<td>1.3</td>
<td>70</td>
<td>130</td>
</tr>
<tr>
<td>Concanavalin A eluates *</td>
<td>15</td>
<td>6.0</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain and G-100 chromatography</td>
<td>2.2</td>
<td>4.8</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Accurate measurements could not be made of the small amounts of protein recovered after this step.
some of the detergent [13]. This fraction is referred to throughout as the 'large' form of \( \gamma \)-glutamyltranspeptidase. No further purification was achieved by G-150 chromatography in buffer A.

To obtain the 'small' form of \( \gamma \)-glutamyl-transpeptidase, the 'large' form was treated with papain (0.4 mg/ml) (Sigma, sodium acetate suspension) at 37°C for 15 min. In later experiments, papain immobilized on a solid matrix was used (Boehringer). No loss in activity is observed with papain treatment. The solution was then passed over a Sephadex G-100 column (80 × 1.6 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5). Fractions of 1.4 ml were collected and assayed for enzyme activity. The most active fractions were pooled and concentrated by pressure dialysis using a PM 10 membrane (Amicon Corp.). When soluble papain was used for making the 'small' form, the G-100 chromatography was repeated to removed residual papain. The results of typical purifications are shown in Tables I and II.

**SDS-Polyacrylamide gel electrophoresis of highly purified tumor \( \gamma \)-glutamyltranspeptidase**

To avoid the use of excessive amounts of purified enzyme in electrophoresis, \(^{125}\)I-labeled enzyme was prepared. Approximately 10 units of purified \( \gamma \)-glutamyltranspeptidase were iodinated by reaction with \(^{125}\)I (carrier free) in the presence of chloramine T [17], and then chromatographed. The radioactivity in the excluded volume of the 0.5 × 30 cm Sephadex G-25 column was collected, dialyzed against several changes of water and lyophilized. This enzymatically inactive material (about 10 000 cpm) was subsequently used for electrophoresis in SDS according to a modification of the method of Laemmli [18]. The developed gels were fixed, stained, and dried [19] and were then placed in a light-protective envelope pressed tightly against a piece of Kodak RP Royal X-OMAT. The film was exposed for about 72 h at \(-70^\circ\)C and was then developed [20].

The developed film showed one major spot. The apparent molecular weight was estimated to be approximately 50 000 by comparison of the mobility with the mobilities of standard radioactive proteins of known molecular weight (kindly provided by Dr. Richard Kirschner). Smaller spots with mobilities corresponding to apparent molecular weights of 85 000, 72 500 and 23 500 were also seen. The major spot (apparent mol. wt. 50 000) is tentatively assumed to correspond to the larger subunit \((M_r 46 000)\) reported for rat kidney [5]. Because our major objective was to verify the high degree of purity of the preparation, no attempt was made to determine whether the spot with the apparent molecular weight of 22 500 might represent the smaller subunit reported [5] for kidney \((M_r 22 000)\).

**Characterization of \( \gamma \)-glutamyltranspeptidase by molecular sieve chromatography**

Before treatment with papain, both mammary and tumor enzyme chromatographed as the 'large' form. This large form was eluted in the void volume of a BioGel A-1.5 m column in an aqueous buffer, but in the presence of 0.2% Triton X-100 the enzyme activity elution peak was shifted to the included volume (Fig. 1). The 'small' form of the enzyme obtained with papain
solubilization showed similar chromatographic properties in the absence or presence of detergent.

The apparent molecular size of both the mammary and tumor enzymes following papain treatment was compared on Sephadex G-100 with Escherichia coli alkaline phosphatase included as an internal standard as seen in Fig. 2. The apparent molecular weight of each preparation is just slightly larger than that of alkaline phosphatase (80 000) and is significantly larger than the value reported [5] for rat kidney (68 000).

**Activity of normal mammary and tumor γ-glutamyltranspeptidase with various γ-glutamyl acceptors**

The enzyme activity of normal mammary and tumor enzyme preparations, both ‘large’ and ‘small’, was measured using a variety of γ-glutamyl acceptors as shown in Table III. The data are expressed as the ratio of the activity measured in the presence of each acceptor to that measured in the absence of any added acceptor, and therefore may be taken as a measure of the stimulation of the reaction by each acceptor. No significant change in substrate specificity
TABLE III

\( \gamma \text{-GLUTAMYLTRANSPEPTIDASE AS MEASURED WITH VARIOUS AMINO ACID ACCEPTORS} \)

The \( \gamma \)-glutamyltranspeptidase activity of each fraction was measured with 2.5 mM \( \gamma \)-glutamyl-p-nitroanilide and 20 mM of each of the amino acids listed. The other common amino acids showed ratios less than 1.5.

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal mammary</td>
</tr>
<tr>
<td></td>
<td>Large</td>
</tr>
<tr>
<td>None</td>
<td>1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.2</td>
</tr>
<tr>
<td>Citrulline</td>
<td>2.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.8</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.0</td>
</tr>
<tr>
<td>Cystine **</td>
<td>1.4</td>
</tr>
<tr>
<td>Glycylglycine</td>
<td>9.5</td>
</tr>
</tbody>
</table>

* Small tumor enzyme (0.4 ml) was treated with neuraminidase (0.2 unit) for 24 h at 37°C. The isoelectric focusing profile at that time showed most of the activity focused in the 10–20 mm region of a 4% polyacrylamide gel with pH 4–6 ampholytes.

** Because of its insolubility, the concentration of cystine used was 0.5 mM.

accompanied papain solubilization. The mammary and tumor enzymes showed similar substrate specificities.

**Kinetic parameters of normal mammary and tumor \( \gamma \)-glutamyltranspeptidase**

Double-reciprocal plots were drawn from data obtained by measuring the units of activity of the mammary and tumor preparations with varying concentrations of \( \gamma \)-glutamyl-p-nitroanilide from 0.5 to 2.5 mM. \( K_m \) values were derived using least-squares analyses to determine the equation of the line that best fit the points. No change in \( K_m \) following papain treatment was seen and no significant difference between normal mammary and tumor enzymes was detected (0.83, 0.94, 0.93, and 0.89 mM\(^{-1}\) for mammary 'large' and 'small' and for tumor 'large' and 'small', respectively).

Because of the lack of a convenient assay using the supposed in vivo substrate, glutathione, the affinity for glutathione was estimated indirectly by its ability to inhibit the reaction with \( \gamma \)-glutamyl-p-nitroanilide. The data indicate classical competitive inhibition patterns for both normal mammary and tumor 'small' enzyme. No significant differences in the calculated \( K_i \) for glutathione was seen (1.44 and 1.00 mM for mammary 'small' and tumor 'small' preparations, respectively).

**Effect of neuraminidase treatment on \( \gamma \)-glutamyltranspeptidase**

Data obtained from isoelectric focusing analysis of these preparations indicated the possibility that the normal mammary and tumor enzymes differed in their sialic acid content [9]. For this reason, the effect of neuraminidase treatment on the enzymatic properties of 'small' tumor enzyme was determined.
Table III shows that this treatment had no significant effect on the γ-glutamyl acceptor specificities. The $K_m$ for γ-glutamyl-$p$-nitroanilide and the $K_i$ for glutathione were determined for the neuraminidase-treated ‘small’ tumor enzyme under the conditions described above for the ‘small’ form of mammary and tumor preparations. This treatment did not appear to have any significant effect on those kinetic parameters ($0.90$ vs. $0.89$ mM$^{-1}$ for $K_m$ and $1.17$ vs. $1.00$ mM for $K_i$).

Discussion

The above method for purification of γ-glutamyltranspeptidase from rat mammary adenocarcinoma differs in many details from those reported previously for the kidney enzyme [5, 22], but yields a product with comparable specific activity and with a high degree of purity according to SDS-polyacrylamide gel electrophoresis. The yields (56%) are very high, a point of importance in isolations from this source. Enzyme from normal mammary tissues was isolated using identical procedures. Both the mammary and tumor enzyme have similar characteristics as those reported for rat kidney enzyme [10, 21, 22] in terms of apparent molecular size, the γ-glutamyl acceptor characteristics, $K_m$ for γ-glutamyl-$p$-nitroanilide, and the transformation of the ‘large’ to the ‘small’ form by proteolysis. This latter transformation is accompanied by a dramatic change in physical (Fig. 2) but not enzymatic properties, as also reported for the rat kidney enzyme [22].

In regards to the enzymatic characteristics of the mammary and tumor enzymes, no significant differences were found, under the conditions used, in the kinetic parameters mentioned above. Treatment with neuraminidase, which markedly altered the isoelectric focusing pattern of the tumor enzyme [9], did not alter the γ-glutamyl acceptor specificity (Table III) or the kinetic parameters. Therefore, on the basis of these in vitro studies, the catalytic properties of the mammary and tumor enzymes and their various isoforms were indistinguishable. It should be noted, however, that these data were collected using the artificial substrate γ-glutamyl-$p$-nitroanilide and artificially high concentrations of acceptor (20 mM). Recent kinetic studies of the kidney enzyme indicate that the hydrolytic reaction may be more important than the transpeptidation reaction at the physiological concentration range of plasma amino acids (0.05—0.5 mM) [23, 24]. Determining the factors necessary for stimulating the hydrolytic activity is necessary for completion of an enzymatic comparison of the normal mammary and tumor enzymes.

The only difference between the normal mammary and tumor enzymatic activities noted was quantitative. The tumor preparations had 6—10 times the specific activity measured for normal mammary preparations. This elevation is not peculiar to transformation, as high specific activities were also measured in the glands from pregnant and lactating rats (Jaken, S. and Mason, M., unpublished results). This indicates that expression of high levels of this activity is a normal response of mammary epithelium under the appropriate hormonal environment. Since the tumor is grown in immature virgin rats, it would seem that the tumor had lost the requirement for hormonal stimuli to produce high levels of the enzyme activity.
The observation that the mammary, tumor, and neuraminidase-treated tumor preparations have similar catalytic properties indicates that the previously observed difference in distribution of isoforms in the tumor enzyme [9] does not confer any metabolic advantage to the tumor cells with respect to altered enzyme function but leaves open the possibility that the structural difference, presumably reflecting increased sialylation [5,9], is related to a more general characteristic of tumor metabolism involving the role of sialic acid in cell surface properties or the regulation of glycoprotein turnover.

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References