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Affinity purification of *Hydra* glutathione binding proteins

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Abstract The association of glutathione (GSH) with putative external chemoreceptors elicits feeding behavior in *Hydra*. In the present study, solubilized membrane proteins were chromatographed on an affinity column of immobilized GSH in order to isolate GSH-binding proteins that may represent the *Hydra* GSH chemoreceptor. The most abundant of the affinity-purified proteins was a triplet of peptides ranging in molecular weight from 24.5–26 kDa. Antiserum generated against the 24.5–26 kDa triplet peptides inhibited GSH-stimulated feeding behavior by 47%, implicating a role for one or more of these peptides in *Hydra* chemoreception.

Key words: Hydra; Coelenterate; Glutathione; Chemoreceptor; Feeding behavior

1. Introduction

Loomis [1] determined that glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) is the physiological activator of a series of feeding behaviors in the freshwater coelenterate, *Hydra*. *Hydra* are bathed with GSH that flows from the coelom of wounded prey. In the presence of GSH, *Hydra* execute tentacle contractions and mouth opening, behaviors which accomodate the ingestion of the prey. Lenhoff [2] developed a quantifiable assay of feeding behavior which consisted of measuring the duration of mouth opening in the presence of GSH. This behavioral assay has yielded extensive information regarding the potency of GSH analogues in stimulating or antagonizing feeding behavior (reviewed in [3]), results which presumably reflect the specificity of the GSH binding site on a putative chemoreceptor. An estimate of the association constant (K_a) for GSH binding (1 μM) was also derived from behavioral assays [2].

More recently, several radioligand binding studies were performed in an attempt to more directly characterize the interaction between the putative GSH chemoreceptor and its agonists or antagonists [4–8]. Two of these binding studies [7,8] investigated the manner in which [^{35}S]GSH associated with a *Hydra* membrane fraction. [^{35}S]GSH binding to the membrane fraction was rapid, reversible and saturable, characteristics which were predicted by results from behavioral assays [3]. In addition, the dissociation constant (K_D) for [^{35}S]GSH binding was estimated as 3.4 μM [8], a value which is in good agreement with GSH concentrations which elicit feeding behavior. It was also shown that saturable, reversible [^{35}S]GSH binding could be solubilized by treatment with 10 mM CHAPS, 10% glycerol, 100 mM KCl [8]. Lack of an effect on the K_D for [^{35}S]GSH binding indicated that GSH-binding proteins were not adversely altered by the detergent-solubilization procedure.

In the present study, solubilized proteins from a *Hydra* membrane preparation were applied to an affinity column which consisted of glutathione linked to agarose through the sulfhydryl moiety of GSH. The sulfhydryl group of GSH is not required for recognition of the GSH molecule by the putative chemoreceptor [3]. Therefore, this approach was used to isolate GSH binding proteins that may play a role in chemoreception.

2. Materials and methods

2.1. Preparation of solubilized membrane proteins

A crude membrane fraction was prepared from 10,000–20,000 *Hydra* by differential centrifugation as previously described [6,7]. Briefly, *Hydra* were homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) containing 10 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 1000 × *g* for 15 min and the supernatant was retained. The pellet was resuspended in Tris-HCl, leupeptin, PMSF, and centrifuged again at 1000 × *g* for 15 min. The supernatant from this centrifugation was combined with the previous supernatant and the combined supernatants were centrifuged at 30,000 × *g* for 15 min, yielding a crude membrane fraction (pellet). This fraction was washed once by resuspension followed by centrifugation (30,000 × *g*, 15 min). The crude membrane fraction was solubilized as described previously [8] in 50 mM Tris, 10 mM CHAPS, 10% glycerol, 100 mM KCl, 10 μg/ml leupeptin, and 0.5 mM PMSF (pH 7.4) for 1 h on ice with intermittent vortexing. The solubilized fraction was centrifuged at 100,000 × *g* for 1 h and the supernatant (solubilized membrane proteins) was retained. Prior to affinity purification, the solubilized preparation was diluted with 50 mM Tris-HCl (pH 7.4), 10 μg/ml leupeptin and 0.5 mM PMSF in order to lower the concentration of CHAPS to 2–5 mM. Preliminary experiments indicated that solubilized membrane proteins retained optimal binding capacity at CHAPS concentrations equal to or lower than 5 mM.

2.2. Inhibition of γ-glutamyl transpeptidase

Hydra membranes contain the GSH-metabolizing enzyme, γ-glutamyl transpeptidase [9,10]. In order to eliminate possible degradation of the GSH-affinity column by this enzyme, the solubilized membrane fraction was treated with two well-established inhibitors of γ-glutamyl transpeptidase, AT-125 [11–15] and borate in combination with serine [16,17]. These inhibitors were used in a previous study [8] to eliminate anomalies in [^{35}S]GSH binding which result from transpeptidase activity. Solubilized membrane proteins were incubated for 40 min on ice with 1.0 mM AT-125, and 5 mM borate/5 mM serine to eliminate γ-glutamyl transpeptidase activity prior to affinity purification.

2.3. Affinity purification of GSH-binding proteins

A buffer composed of 50 mM Tris, 1 mM CHAPS, 1% glycerol, 100 mM KCl, 10 μg/ml leupeptin, 0.5 mM PMSF, and 5 mM borate/5 mM serine (pH 7.4) was used to equilibrate a glutathione-agarose affinity column (10 ml bed volume) prior to loading the solubilized fraction onto the column. Borate/serine was included in the equilibration buffer because, unlike AT-125, borate/serine is a reversible inhibitor of γ-glutamyl transpeptidase [17] and must be continuously present to eliminate enzyme activity. After treating the solubilized fraction with

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the γ-glutamyl transpeptidase inhibitors described above, this fraction was loaded onto the equilibrated GSH-agarose affinity column. The solubilized fraction was allowed to enter the column and then solvent flow was interrupted for 1 h to maximize the association with GSH. The column was washed with approximately 10 column vols. of equilibration buffer. GSH-binding proteins were eluted with 50 mM GSH, 1% glycerol, 1 mM CHAPS, 10 mM KCl and 50 mM Tris (pH 7.4). During the elution, the solvent flow was again stopped for 1 h to maximize the dissociation of proteins from the column. All chromatography steps were performed at 4°C.

Because it has been suggested that Ca^{2+} may be required for GSH association with the putative GSH chemoreceptor [3,7], the above purification procedure was also performed with the addition of 1 mM Ca^{2+} to all buffers. The addition of Ca^{2+} to the purification procedure did not alter the profile of peptides resulting from affinity purification.

2.4. Electrophoresis of GSH-binding proteins

The eluted fraction from the GSH affinity column was desalted on 10 DG desalting columns (Bio-Rad) and lyophilized. Lyophilized samples were dissolved in SDS-PAGE sample buffer (40 mM Tris (pH 6.8), 8% glycerol, 1% SDS, 0.003% Bromophenol blue and 5% β-mercaptoethanol), then boiled for 3–5 min. Samples were electrophoresed on a 10% SDS-polyacrylamide gel [18], followed by silver-staining of the gel (Bio-Rad).

2.5. Generation of antiserum and immunoblotting

An affinity-purified fraction was obtained from 50,000 *Hydra* as described above. This fraction was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was stained for 40 min with 2% Coomassie blue dissolved in distilled, deionized H_2O. The gel was destained with several changes of distilled, deionized H_2O until bands became visible [19]. Bands were excised from the gel, emulsified with Freund's incomplete adjuvant, then injected into rabbits. Four samples were injected; (i) a single 48 kDa band, (ii) a doublet composed of 27.5 and 27 kDa peptides, (iii) a triplet composed of 24.5, 25 and 26 kDa peptides, and (iv) a single 20 kDa band (see section 3, Fig. 2). A second injection of antigen was performed 16 days after the first injection and serum was collected 8 days after the second injection.

The detection of anti-*Hydra* immunoglobulins in rabbit serum was accomplished by immunoblotting [20]. The eluted fraction from the GSH affinity column was electrophoresed on a 10% SDS-polyacrylamide gel, then transferred to nitrocellulose in Towbin buffer [21] using a semi-dry transfer apparatus (Bio-Rad). Nitrocellulose strips were blocked for 1 h with 3% BSA plus 0.15% non-fat dry milk dissolved in Tris-buffered saline (TBS, pH 7.0), then incubated with either preimmunized or immunized rabbit serum (1:100 dilution) overnight. After several washes with TBS, strips were incubated for 12 h with goat anti-rabbit IgG linked to colloidal gold particles (1:100 dilution). The colloidal gold signal was intensified by silver enhancement following a procedure recommended by the vendor of silver enhancement reagents (Zymed).

2.6. Hydra cultures and assay of feeding behavior

Specimens of *Hydra attenuata* were cultured in BVC solution [22] and fed with *Artemia nauplii* as previously described [6,7]. *Hydra* were starved for 48 h prior to the behavioral assay. Each treatment group (seven *Hydra*) was preincubated with either preimmunized rabbit serum, or serum collected from rabbits immunized with affinity-purified GSH-binding proteins (see Fig. 2). Serum was added to BVC solution in a 1:50 dilution. Following incubation in serum/BVC solution for 10 min at room temperature, GSH was added to the serum/BVC solution to yield a final GSH concentration of 2 μM. After the addition of GSH to the medium, *Hydra* were observed to open their mouths within 2–3 min. The duration of the mouth opening response was subsequently measured.

2.7. Materials

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Calbiochem (San Diego, CA). Silver staining reagents were obtained from Bio-Rad Laboratories (Richmond, CA). Goat anti-rabbit/colloidal gold IgG and silver enhancement solutions were obtained from Zymed (San Diego, CA). All other reagents including GSH-agarose and AT-125 (Acivicin) were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

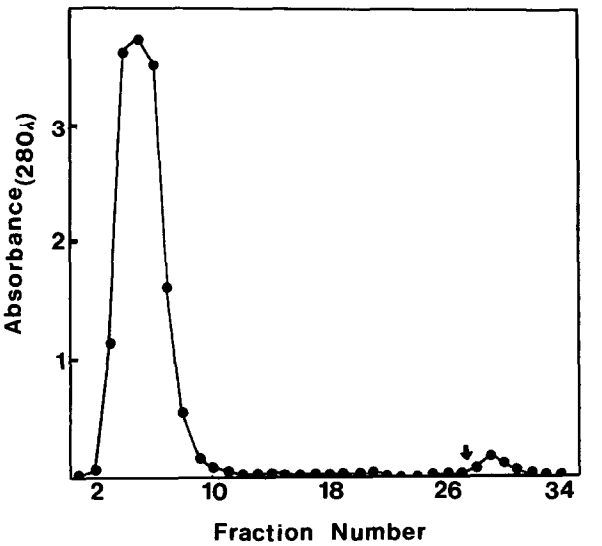

Fig. 1. Affinity purification of *Hydra* GSH-binding proteins. A CHAPS-solubilized membrane fraction was loaded onto a 10 ml GSH affinity column. After several washes with equilibration buffer to remove unbound proteins, proteins which bound the column were eluted with 50 mM GSH (arrow). Peak fractions (28–30) were combined.

3. Results

A *Hydra* membrane preparation was solubilized in 10 mM CHAPS, 10% glycerol, 100 mM KCl, then applied to a GSH-affinity column. After washing the column with equilibration buffer to remove unbound proteins, proteins which bound the column were eluted with buffer containing 50 mM GSH (Fig. 1). Peak fractions (fractions 28–30) were pooled. The eluted fraction was electrophoresed on a 10% SDS-polyacrylamide gel and bands were visualized by silver-staining (Fig. 2). The following bands were observed: a single band at 48 kDa, a doublet at 27.5 and 27 kDa ('27 kDa doublet'), a triplet at 24.5, 25 and 26 kDa ('25 kDa triplet') and a single band at 20 kDa. Additionally, the eluted material was electrophoresed on a 5% SDS-polyacrylamide gel (not shown) in order to insure against the undetected presence of large molecular weight peptides. No bands greater than 48 kDa were detected on the 5% gel.

Affinity-purified peptides were excised from polyacrylamide gels and injected into rabbits in order to generate polyclonal antibody against the peptides. Four samples were excised from the gel and injected into rabbits: (i) the 20 kDa single band, (ii) the 25 kDa triplet, (iii) the 27 kDa doublet, and (iv) the 48 kDa single band (see Fig. 2). The detection of anti-*Hydra* immunoglobulins in rabbit serum was accomplished by immunoblotting affinity-purified peptides that had been electrophoresed, then transferred to nitrocellulose. As shown in Fig. 3, anti-*Hydra* immunoglobulins were detected in serum collected from a rabbit immunized with the 25 kDa triplet peptides. Immunoblotting experiments were not successful in identifying anti-*Hydra* immunoglobulins in the other serum samples.

To assess the potential role of affinity-purified peptides in chemoreception, sera collected from rabbits immunized with the affinity-purified peptides were tested for effects on GSH-stimulated mouth opening behavior. Whole animals were preincubated with either normal rabbit serum or with one of the

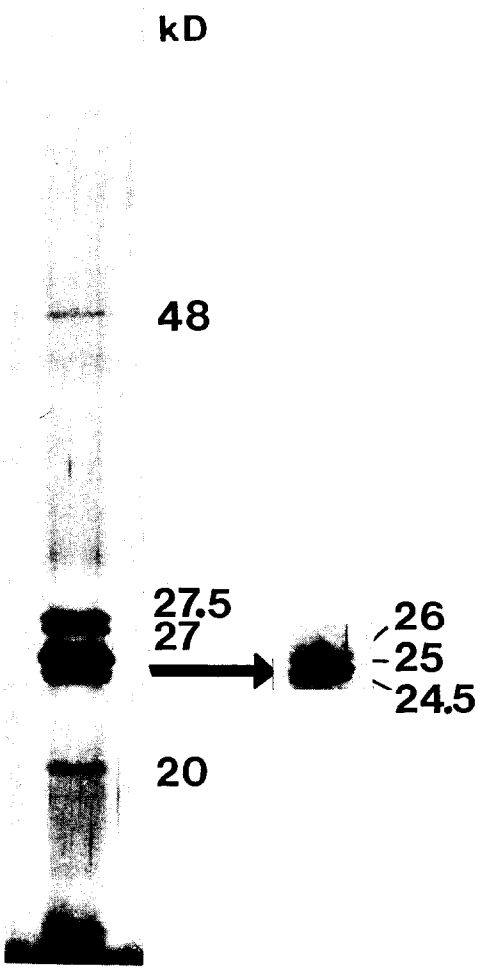

Fig. 2. Electrophoresis of affinity-purified peptides. The eluted fraction (pooled fractions 28–30) from GSH affinity chromatography was desalted, then lyophilized. The lyophilized fraction was resuspended in SDS-PAGE sample buffer, boiled for 5 min, then electrophoresed on a 10% polyacrylamide gel. Detection of bands was accomplished by silver staining.

four immunized serum samples described above. Animals were then stimulated with glutathione and the duration of GSH-induced mouth opening behavior was measured. Of the four immunized serum samples, only antiserum against the 25 kDa triplet peptides altered the duration of mouth opening behavior relative to animals incubated with preimmunized serum. Animals exposed to anti-25 kDa antiserum exhibited a mouth opening response lasting 32 min as compared with the 60 min response of control animals (Table 1). This corresponds to a 47% reduction in the duration of feeding behavior. In contrast, animals incubated with the other three immunized serum samples demonstrated a response lasting 58–70 min (not shown). Neither the preimmunized serum nor any of the immunized serum samples had any effect on *Hydra* behavior in the absence of GSH.

4. Discussion

It was previously established that a CHAPS-solubilized membrane fraction bound [^{35}S]GSH in a saturable, reversible manner [8]. It was thus assumed that detergent-solubilized GSH-binding proteins retained adequate binding capability to allow purification on a GSH affinity column. Application of the CHAPS-solubilized membrane fraction to the GSH column resulted in the isolation of several peptides in the 20–48 kDa range (Fig. 2). The electrophoretic conditions employed (5% and 10% polyacrylamide) in evaluating the molecular weights of affinity-purified peptides allowed the detection of peptides ranging in molecular weight from approximately 10 to 300 kDa. Under these conditions, no GSH-binding proteins greater than 48 kDa were detected. A 220 kDa peptide was proposed to represent a GSH receptor which mediates tentacle contraction in *H. japonica* [23]. The relationship between the 220 kDa protein and the peptides isolated in the present work is not clear; however, different approaches, including the target behavior, and different species of *Hydra* were used to isolate candidate receptor proteins in the two studies. Apparent discrepancies in results may arise from these factors.

Antiserum against the affinity-purified, 25 kDa triplet peptides significantly inhibited GSH-stimulated feeding behavior (Table 1). This is strong evidence that one or more of these peptides plays a role in chemoreception. It is not known to what extent the triplet peptides are related. These peptides may represent different phosphorylation or degradation states of the same protein, or they may be distinct proteins altogether. The 27 kDa doublet peptides appear to be unrelated to the 25 kDa peptides because antiserum against the 25 kDa triplet peptides failed to recognize the 27 kDa peptides in immunoblotting experiments (Fig. 3).

None of the serum samples had any effect on feeding behavior in the absence of GSH. However, all of the animals exposed to rabbit serum, including preimmunized serum, demonstrated an attenuated GSH-induced feeding response as compared with animals which were not exposed to any type of serum (data not shown). This is consistent with earlier reports which indicated that serum components such as growth factors have an inhibitory effect on GSH-stimulated feeding behavior [24]. In the present study, preincubation of *Hydra* with all serum samples other than the anti-25 kDa antiserum resulted in a feeding reponse lasting from 58–70 min. The small variation in the duration of feeding behavior in animals treated with serum

Table 1
Effect of immunized serum on GSH-stimulated mouth opening behavior

Type of serum	Duration of mouth opening (min)
Normal preimmune serum	60 ± 6[a]
Serum from immunization with the 24.5, 25 and 26 kDa peptides	32 ± 5[b]
Serum from immunization with the 27 and 27.5 kDa peptides	61 ± 6[c]

Hydra were starved for 2 days prior to the behavioral assay. Treatment groups (seven *Hydra* each) were incubated for 10 min with a 1:50 dilution of sera in BVC solution. Sufficient GSH was then added to bring the final GSH concentration to 2 μM and the duration of mouth opening behavior was measured. Values represent the mean and ±S.E.M. for each treatment group.

[a]Serum collected prior to immunization.

[b]This mean is significantly different from preimmunized serum mean ($P < 0.05$).

[c]Because no antibody was detected in this sample, this serves as an adjuvant control.

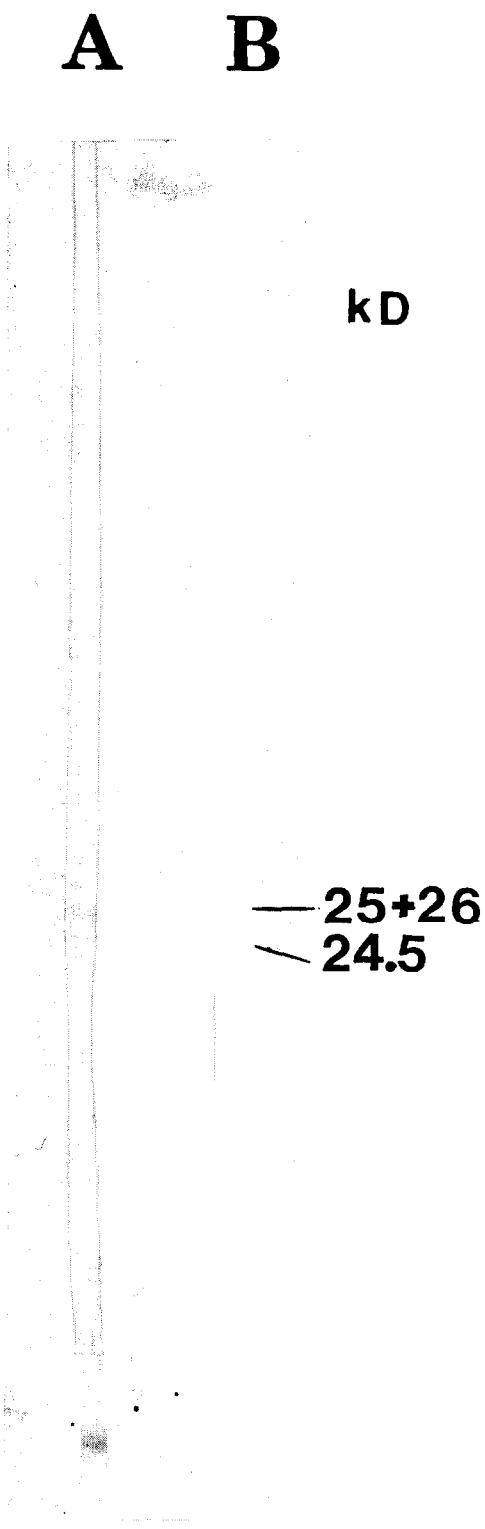

Fig. 3. Immunoblotting of affinity-purified peptides. Affinity-purified peptides were electrophoresed, then transferred to nitrocellulose in Towbin buffer [21]. After a blocking step, peptides were incubated with either normal rabbit serum (lane A) or serum from a rabbit innoculated with the 25 kDa affinity-purified triplet peptides (lane B). The nitrocellulose strips were then incubated with goat anti-rabbit IgG which was complexed to colloidal gold. The colloidal gold signal was intensified by silver enhancement.

samples other than the anti-25 kDa antiserum suggests that, under our conditions, the 47% inhibition in feeding behavior effected by the anti-25 kDa antiserum is most likely due to the presence of immunoglobulins, and not due to fluctuations in the level of other serum components.

Anti-*Hydra* immunoglobulins were not detected in the serum of rabbits immunized with the 20, 48 or 27 kDa doublet peptides. Because the 20, 48 and 27 kDa peptides were less abundant than the 25 kDa affinity-purified peptides, less antigen was available for immunization. It is likely that the amount of the 20, 48 and 27 kDa peptides injected into rabbits was insufficient to induce a robust immune response. At least 100,000 *Hydra* were used in generating antibody against the affinity-purified peptides. Further attempts to obtain antibody against the 20, 48 and 27 kDa peptides were not performed due to limitations in generating greater numbers of *Hydra* and due to the success of analyses with the 25 kDa peptides.

Because no antibody was detected in sera collected from rabbits immunized with the 20, 48 or 27 kDa peptides, the lack of effect on feeding behavior by these serum samples is not meaningful with regard to negating a role for these peptides in chemoreception. The possibility remains that any of these peptides could represent subunits of the native GSH receptor. The affinity column approach employed in the present study does not necessarily separate GSH-binding proteins from other proteins that bind with high affinity and specificity to GSH-binding proteins. The *Hydra* GSH receptor may be composed of subunits of such proteins. The anti-25 kDa antiserum generated in the present study is expected to be useful in further investigations directed at resolving the native structure of the GSH receptor.

In conclusion, *Hydra* GSH-binding proteins were isolated by affinity chromatography with the intent to identify proteins which may be candidates for the GSH chemoreceptor. Antiserum against a 25 kDa triplet of affinity-purified peptides inhibited feeding behavior by 47%, suggesting that one or more of the triplet peptides may represent or comprise some portion of the GSH receptor.

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