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## **Binding of *Aedes aegypti* Trypsin Modulating Oostatic Factor (*Aea*-TMOF) to its receptor stimulates phosphorylation and protease processing of gut-membrane proteins**

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**Abstract:** The binding of TMOF to its gut receptor was followed by incubating guts removed from male and female *Aedes aegypti*. TMOF at physiological concentrations, in the presence of [ $\gamma^{32}$ P]ATP, causes phosphorylation and release of gut-membrane protein (45 kDa) that is further processed by proteolysis. In the presence of protease inhibitors only the 45 kDa protein was released. The phosphorylation and processing of the 45 kDa protein does not happen in the absence of TMOF. Both larvae and adult guts release the protein in the presence of TMOF. Male *Ae. aegypti* do not synthesize trypsin in their gut and do not release the 45 kDa protein in the presence of TMOF because a TMOF receptor is probably absent. Homogenized guts do not release the 45 kDa protein, indicating that the protease processing or the ecto-protein kinase activity is probably reduced after breaking the tissue. The 45 kDa phosphorylated protein can be dephosphorylated by alkaline phosphatase and protein phosphatase, indicating that the phosphate group is covalently linked to either a serine or a tyrosine moiety. This is the first report that shows that in insects, binding of a peptide hormone activates its receptor by proteolysis.

**Keywords:** mosquitoes, *in vitro*, TMOF gut receptor, electrophoresis, fluorography

## INTRODUCTION

The mosquito ovary serves as a rich source of “oostatic hormone” [1]. The hormone appears to affect the activity of those midgut cells that synthesize trypsin, but not ovarian or endocrine tissues [2]. The hormone is not species-specific, as injection of the *Aedes aegypti* hormone inhibits egg development and trypsin biosynthesis in *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* [2]. Because the target tissue of the hormone is the mosquito midgut and not the ovary or the brain, the hormone was named “Trypsin Modulating Oostatic Factor” (TMOF). The hormone has been purified, sequenced and characterized by means of mass spectroscopy as an unblocked decapeptide (YDPAPPPPP) [3]. Various synthetic peptide analogues possess TMOF activity [3-5]. NMR analyses [6] confirm computer-modeling suggestions that the polyproline portion of TMOF in solution is a left-handed alpha helix [3, 5]. TMOF does not act as a classical trypsin inhibitor (e.g. TLCK, TPCK and Soybean trypsin inhibitor) that binds to the active site of serine proteases and prevents protein hydrolysis. Instead, TMOF binds to a specific gut epithelial cell receptor before stopping trypsin biosynthesis [3, 7].

Specific TMOF binding sites on the midgut epithelial cells proliferate after the blood meal, as visualized by cytoimmunochemical staining at 24 and 72 h after the blood meal. Two classes of binding sites are present on the gut membrane: high affinity ( $K_{d1} = 4.6 \pm 0.7 \times 10^{-7}$  M;  $K_{assoc} = 2.2 \times 10^6$  M<sup>-1</sup>;  $B_{max} = 0.1$  pmole/gut) and low affinity ( $K_{d2} = 4.43 \pm 1 \times 10^{-6}$  M;  $K_{assoc} = 2.3 \times 10^5$  M<sup>-1</sup>;  $B_{max} = 0.2$  pmole/gut). The total binding sites for high and low affinity classes of TMOF per gut approximate  $6.3 \times 10^{10}$  and  $1.1 \times 10^{11}$  sites, respectively [7]. Thus, at 24 h, when trypsin biosynthesis is at its highest level, the follicular epithelium of the ovary begins to synthesize and release TMOF into the hemolymph. Maximum synthesis of TMOF occurs at approximately 33 h to 36 h after the blood meal and the synthesis declines afterwards, and stops at 48 h [7]. Once in the hemolymph, TMOF binds to the gut epithelial cells and signals them to cease trypsin biosynthesis, which rapidly declines and stops at 48 h [1, 3, 5, 7-9].

The effect of TMOF on the trypsin gene was first studied in *Neobellieria*. After injecting TMOF into these flies, the biosynthesis of trypsin mRNA was followed using Northern blot analysis [10]. Feeding these flies a liver meal causes degradation of the endogenous trypsin early mRNA, and synthesis of a new mRNA that corresponds to late trypsin biosynthesis associated with post meal digestion. In flesh flies injected with *Neobellieria* TMOF ( $10^{-9}$  M), the early mRNA does not disappear, and the late mRNA that is synthesized is not translated. TMOF, therefore, controls the translation of late trypsin mRNA, as

would be expected for a hormone that is released after trypsin mRNA has been transcribed [10].

Because of the insecticidal affects of TMOF and the potential of the hormone to affect larval mosquito and several Lepidoptera larvae, we studied, *in vitro*, the biochemical events that occur after binding of the hormone to its gut receptor. This report shows, for the first time, that TMOF binding to its gut receptor induces gut membrane phosphorylation and protease activation which are probably the first steps in a signal transduction pathway that controls the translation of the trypsin message in the gut of many insects.

## MATERIALS AND METHODS

### Insects

Larval *Ae. aegypti* were reared at 27 °C on a diet of Brewer's yeast and lactalbumin (1:1) under 16:8 h light:dark cycle. Adults were fed on 5% sucrose or on chicken blood. Guts were dissected out in saline from 4<sup>th</sup> instar larvae, newly emerged adults (1 to 2 days old), or from older adults (4-10 days olds).

### TMOF and Chemicals

TMOF was synthesized by Synpep (California, USA), purified by HPLC and analyzed by mass spectrometry (purity  $\geq 98\%$ ), dried and stored at -80 °C until used. Proteinase inhibitor cocktail (Sigma, St. Louis MS, USA) was dissolved in 10 mL water and diluted 1:10 with membranes incubation mixtures. All other chemicals were purchased from Fisher Scientific (Atlanta, GA, USA). Radioactive ATP [ $\gamma$  P<sup>32</sup>] (6,000 Ci /mmol) was purchased from Perkin Elmer Biosciences (USA) and was used within 10 days after arrival.

### Gut preparation and phosphorylation

Adults and larval guts (5 to 10 guts) were dissected under a dissecting microscope, washed in saline and the guts were transferred into Eppendorf tubes (1.5 mL) containing 600  $\mu$ L of phosphate buffer 0.2 M, 150 mM NaCl, pH 7.4 (PBS) and proteinase inhibitor Cocktail (Sigma, St Louis MS, USA). The guts were washed 4 times by centrifugation at 5,000 rpm in an Eppendorf centrifuge, and the pellet was resuspended in 300  $\mu$ L PBS, pH 7.4 with or without proteinase inhibitor cocktail. To the resuspended gut preparation, TMOF (50  $\mu$ g) and 1  $\mu$ L [ $\gamma$ P<sup>32</sup>]ATP (10  $\mu$ Ci, 6,000 Ci/mmmole) were added. The tubes were briefly vortexed and incubated at room temperature (25 °C) for 15 min. Following incubation, the reaction tubes were centrifuged at 13,000 rpm, the supernatants

removed and stored frozen at  $-20\text{ }^{\circ}\text{C}$  before drying by SpeedVac for analysis by SDS-PAGE. Dried supernatant proteins were dissolved in 2% mercaptoethanol (ME) and 2% SDS.

### **Membrane proteins extraction**

In order to extract all the phosphorylated membrane proteins, 300  $\mu\text{L}$  PBS containing 1% CHAPS was added to the reaction mixture containing phosphorylated guts. After a short incubation of 5 min at room temperature with constant vortexing of the incubation tubes, the guts were homogenized with a glass homogenizer (Kontes) and centrifuged at 13,000 rpm. The supernatants were removed, dried by SpeedVac and stored at  $-20\text{ }^{\circ}\text{C}$ .

### **SDS-PAGE and autoradiography**

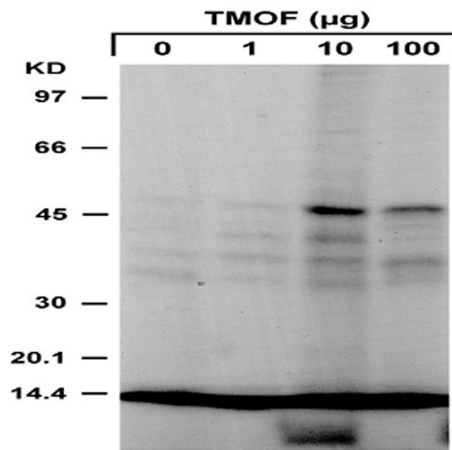
Samples that were dried by SpeedVac, were rehydrated in 100  $\mu\text{L}$  of electrophoresis sample solution (62.5 mM Tris-HCl, pH 6.8, 20% Glycerol, 2% SDS, 5%  $\beta$ -ME and 0.003% bromophenol blue) and heated at  $100\text{ }^{\circ}\text{C}$  for 5 min. Samples were resolved by 10% polyacrylamide gel electrophoresis [11] using Bio-Rad Protean II system. The gel was stained using Coomassie Brilliant Blue and destained for 24 h. The destained gel was dried under vacuum in a gel dryer (BioRad, CA, USA) and exposed to X-Ray film at  $-70\text{ }^{\circ}\text{C}$  for 3 to 6 days.

## **RESULTS AND DISCUSSION**

### **Effect of TMOF on *Aedes aegypti* guts**

To find out if, *in vitro*, TMOF induces phosphorylation, forty guts from sugar fed females were dissected under a microscope, and washed 3 times in saline by centrifugation in Eppendorf tubes. Four-groups of 10 guts each were incubated in PBS, pH 7.4 (200  $\mu\text{L}$  with different concentrations of TMOF ( $4.8 \times 10^{-6}$  M,  $4.8 \times 10^{-5}$  M and  $4.8 \times 10^{-4}$  M) in the presence of [ $\gamma^{32}\text{P}$ ]ATP (1  $\mu\text{L}$ ) for 2 h at  $24\text{ }^{\circ}\text{C}$ . TMOF concentration of  $4.8 \times 10^{-5}$  M is the physiological concentration of TMOF in the hemolymph of female *Ae. aegypti* 30-33 h after the blood meal (Borovsky, unpublished observations). Controls were incubated without TMOF. After incubation, the reaction mixture was centrifuged and the supernatants and guts were analyzed on 10% polyacrylamide gels using SDS-PAGE. The gels were stained with Coomassie Brilliant Blue, dried, and exposed to X-ray film for 3 days at  $-80\text{ }^{\circ}\text{C}$ . Guts that were incubated with TMOF ( $4.8 \times 10^{-5}$  M and  $4.8 \times 10^{-4}$  M) released a phosphorylated protein ( $M_r$  45,000) into the incubation media. Low phosphorylation was observed when TMOF was absent or at

a concentration of  $4.8 \times 10^{-6}$  M. At high TMOF concentration ( $4.8 \times 10^{-4}$  M) the intensity of the phosphorylated band was reduced several-fold. These results indicate that binding of TMOF to its gut receptor causes protein phosphorylation, probably by an ecto-protein kinase [12], and the phosphorylated protein is cleaved from the gut membrane by a protease (Figure 1). The phosphorylated band was further processed by proteolysis into 3 smaller bands (Figure 1). No phosphorylated proteins were found when the guts were extracted with SDS and ME and analyzed by SDS PAGE. These results indicate that TMOF binds a gut receptor and induces phosphorylation of a gut protein, which is cleaved by a protease and released from the gut.

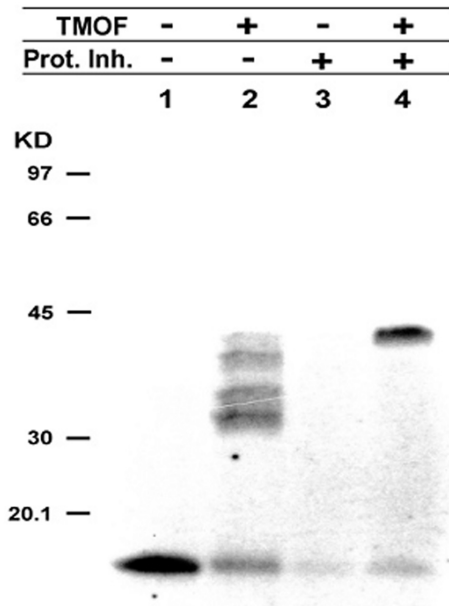


**Figure 1.** *Ae. aegypti* guts were incubated in the presence of TMOF: 0  $\mu\text{g}$ , 1  $\mu\text{g}$  ( $10^{-6}$  M), 10  $\mu\text{g}$  ( $10^{-5}$  M) and 100  $\mu\text{g}$  ( $10^{-4}$  M) and  $[\gamma^{32}\text{P}]\text{ATP}$ . Following incubations, the media were analyzed by SDS PAGE.

### Effect of protease inhibitors

To find out if the proteolytic processing of the phosphorylated protein into smaller moieties can be stopped, guts were incubated for 15 min in PBS with TMOF ( $4.8 \times 10^{-5}$  M) or without TMOF and with  $[\gamma^{32}\text{P}]\text{ATP}$  and protease inhibitor cocktail (Sigma). The cocktail contains inhibitors for serine proteases, metalloproteases, aminopeptidases, alanyl peptidases and cysteine proteases. SDS-PAGE analysis showed that a single phosphorylated protein ( $M_r$  45,000) was detected when guts were incubated with TMOF and protease inhibitor cocktail (Figure 2, lane 4). In the absence of protease inhibitors, four phosphorylated protein bands were detected. These results indicate that the gut-phosphorylated protein ( $M_r$  45,000) is processed by protease(s) that are sensitive to the protease inhibitors cocktail.

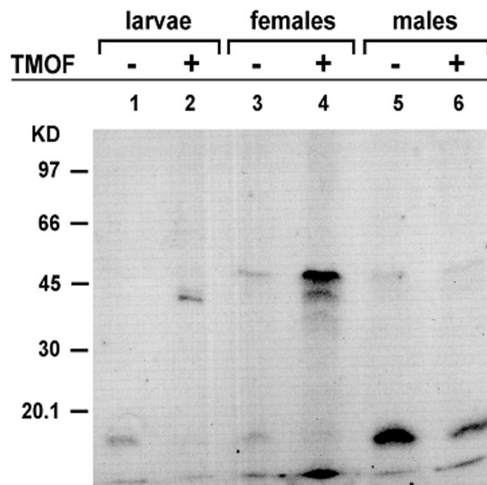
On the other hand, the initial proteolysis of the phosphorylated gut protein was insensitive to the protease inhibitor cocktail. No phosphorylation was observed in the absence of TMOF or in the presence of the protease inhibitor cocktail alone (Figure 2 lanes 1 and 3). These results indicate that the phosphorylation event is TMOF dependent, and the protease that cleaves the phosphorylated gut protein is probably anchored on the surface of the gut in close proximity to an ecto-protein kinase and the receptor. A second possibility is that the released protein ( $M_r$  45,000) is part of the TMOF receptor, which is phosphorylated and cleaved after TMOF binds the gut's receptor. A third possibility is that the phosphorylated protein is not cleaved from the gut membrane but is secreted and phosphorylated after the binding of TMOF to its gut receptor, however, we have never observed a Coomassie Brilliant Blue stained protein band secreted into the medium if guts were incubated only with or without TMOF. Thus, the binding of TMOF to its receptor in concert with gut phosphorylation and proteolysis releases the phosphorylated 45 kDa protein.



**Figure 2.** Incubation of mosquito guts with TMOF and protease inhibitor cocktail. Female *Ae. aegypti* guts were incubated with  $[\gamma^{32}\text{P}]\text{ATP}$  in the presence and absence of protease inhibitors or TMOF and analyzed by SDS PAGE.

### Effect of TMOF on larval and adult guts

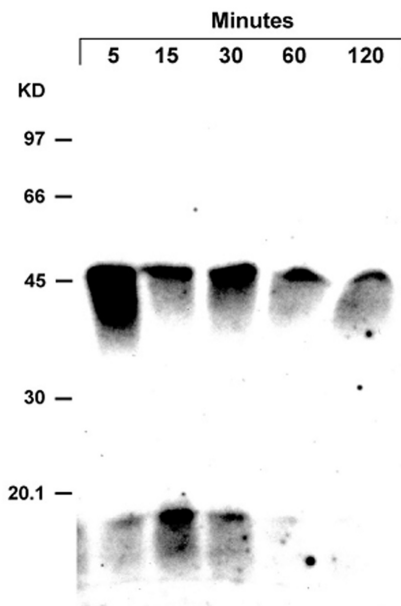
To find out the effect of TMOF on larvae and newly emerged adult male and female *Ae. aegypti*, guts (5 per group) were collected from 4<sup>th</sup> instar larvae, and newly emerged males and females. The guts were washed 3 times by centrifugation and incubated at 24 °C with or without TMOF ( $4.8 \times 10^{-5}$  M) in the presence of protease inhibitor cocktail, and [ $\gamma^{32}$ P]ATP. Following incubation, the guts were centrifuged and the pellet extracted with 1% CHAPS. The extracted gut proteins were combined with the supernatants and analyzed by SDS PAGE. Guts that were incubated with TMOF released phosphorylated protein of about 45 kDa into the medium when female and larval guts were assayed (Figure 3 lanes 2 and 4). No phosphorylated protein of 45 kDa was detected when male guts were incubated with TMOF (Figure 3, lane 6). CHAPS did not extract additional phosphorylated proteins from the gut's pellet indicating that the proteins that are phosphorylated are on the surface of the gut and are released during the incubation by a protease that is probably anchored in close proximity. Because adult male *Aedes aegypti* do not take a blood meal and do not synthesize trypsin, their guts lack a TMOF receptor. No phosphorylation and release of phosphorylated protein (45 kDa) was detected in males even after extraction with CHAPS (Figure 3, lane 6). In the absence of TMOF, larval and adult male and female guts were not phosphorylated, and no phosphorylated protein ( $M_r$  45,000) was found even after extraction with CHAPS (Figure 3, lanes 1, 3 and 5).



**Figure 3.** Incubations of *Ae. aegypti* guts removed from larvae, females and males with [ $\gamma^{32}$ P]ATP in the presence and absence of TMOF. After incubations, the media and guts were analyzed by SDS PAGE.

### Time-course of gut phosphorylation

Female *Ae. aegypti* guts (5 per group) were incubated in PBS, pH 7.4 containing protease inhibitors cocktail, TMOF ( $4.8 \times 10^{-5}$  M) and [ $\gamma^{32}$ P]ATP for 5, 15, 30, 60 and 120 min, to follow gut phosphorylation. After incubation, the media were centrifuged, and the supernatants collected and the pellets containing the guts were extracted with CHAPS. The extracts and supernatants from each incubation period were combined and analyzed by SDS PAGE. Maximum labeling of the gut protein was observed after 5 min and the labeling declined several fold after 2 h incubation (Figure 4). One explanation is that during longer incubation periods of 60 to 120 min the gut releases phosphatases (e.g. alkaline phosphatases, Ser/Thr phosphatases PP1 and PP2a, Mg<sup>+2</sup> and Ca<sup>+2</sup> dependent phosphatases, or Y-phosphatases) that partially dephosphorylate the gut protein (Figure 4). These results indicate that phosphorylation and proteolysis events are rapid, and optimal phosphorylation and protein release are observed in 5 to 30 min (Figure 4).

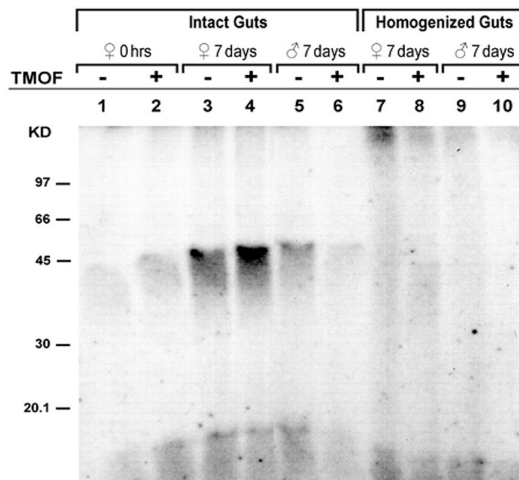


**Figure 4.** Time course of gut protein phosphorylation. Guts from female *Ae. aegypti* were incubated with TMOF and [ $\gamma^{32}$ P]ATP for 5 to 120 min and analyzed for the release of phosphorylated proteins by SDS PAGE.



### Comparison between homogenized and intact guts

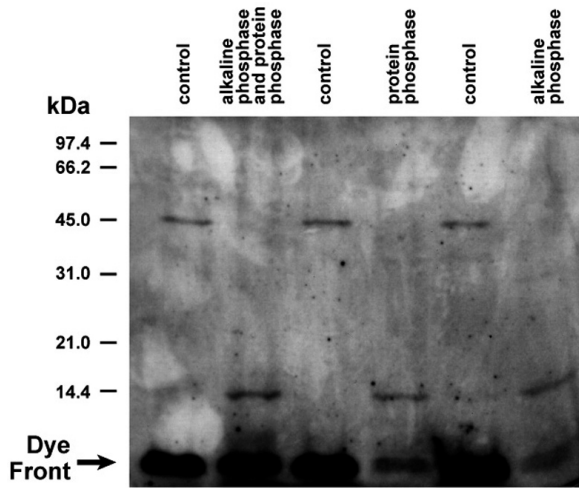
To find out if gut membranes prepared from intact guts can be phosphorylated in the presence of TMOF and release a 45 kDa protein, *Ae. aegypti* guts (5 per group) were lightly homogenized in a glass homogenizer containing PBS, pH 7.4 and protease inhibitor cocktail. The homogenate was centrifuged, and the pellet containing gut membranes was collected and incubated with physiological concentration of TMOF ( $4.8 \times 10^{-5}$  M) and  $[\gamma^{32}\text{P}]\text{ATP}$  for 5 min. The results were compared with incubations of intact guts from newly emerged and 7 days old female and male *Ae. aegypti* (controls). Intact guts from 7 days old females released a phosphorylated protein (45 kDa) in the presence of TMOF, whereas intact guts of newly emerged females or homogenized gut membranes did not release the phosphorylated protein in the presence or absence of TMOF (Figure 5). These results indicate that phosphorylation by ecto-protein kinase and proteolysis can be observed only with intact guts of older females. Thus, TMOF receptor is probably absent in newly emerged females (Figure 5 lane 2) and is re-synthesized after adult eclosion. Gut membranes that are homogenized lose their capability to phosphorylate and release the 45 kDa protein, because homogenization probably causes a release or denaturation of the ecto-protein kinase and the protease. No phosphorylated proteins were found in the membranes when the membranes were treated with CHAPS and assayed using SDS PAGE (results not shown).



**Figure 5.** Phosphorylation of intact and homogenized guts of females and males *Ae. aegypti*. Intact guts and gut homogenates were incubated in the presence and absence of TMOF with  $[\gamma^{32}\text{P}]\text{ATP}$  and analyzed by SDS PAGE.

### Dephosphorylation of the 45 kDa protein

The incubation of guts in the presence of [ $\gamma^{32}\text{P}$ ]ATP and TMOF releases a 45 kDa phosphorylated protein (Figures 1-5). To show that the radioactively label phosphate is covalently bound to the protein and that the protein is not an ATP binding protein, *Ae. aegypti* guts (10 per group; 3 groups) were incubated in PBS, pH 7.4 buffer containing protease inhibitor cocktail, TMOF ( $4.8 \times 10^{-5}$  M) and [ $\gamma^{32}\text{P}$ ]ATP for 15 min. After incubation, the media from each group were divided in half. One half was incubated with alkaline phosphatase (10 units; Sigma), or with protein phosphatase-1 catalytic subunit (10 units; Sigma), or in the presence of both alkaline and protein phosphatases (5 units, respectively) for 1 h at room temperature. The other half or the reaction mixtures (controls) were incubated for 1 h at room temperature in the presence of heat inactivated alkaline phosphatase, or protein phosphatase-1 catalytic subunit, or both alkaline phosphatase and protein phosphatase-1. The reaction media were then analyzed by 10% PAGE-SDS. The gels were stained with Coomassie Brilliant Blue, dried and exposed to X-ray film at  $-80$  °C for 24 h. In all the control groups a phosphorylated band at 45 kDa and a heavy phosphorylated band that ran with the dye front (free [ $\gamma^{32}\text{P}$ ]ATP) was detected (Figure 6). However, when the control groups were incubated with alkaline phosphatase, protein phosphatase-1 or both, the phosphorylated band at 45 kDa disappeared and a new band that ran at about 14.4 kDa appeared. This is due not to protease activity, (all reactions were incubated in the presence of protease inhibitor cocktail) but to the slower movement of free [ $^{32}\text{PO}_4$ ] that was liberated from the dephosphorylated protein as compared with the faster migration of unreacted [ $\gamma^{32}\text{P}$ ]ATP that was detected in the dye front (Figure 6). These results indicate that the 45 kDa protein moiety is phosphorylated and it is not an ATP binding protein.



**Figure 6.** Dephosphorylation of gut membrane protein (45 kDa) with alkaline phosphatase and protein phosphatase-1. Guts were incubated with TMOF and  $[\gamma^{32}\text{P}]\text{ATP}$  and phosphorylated proteins released into the incubation medium were dephosphorylated with alkaline phosphatase, protein phosphatase-1 or both enzymes. Controls were incubated with heat inactivated enzymes. Bands at 14.4 kDa represent released  $[\text{32P}]$  that migrates slower than the unreacted  $[\gamma^{32}\text{P}]\text{ATP}$  which runs with the dye front.

### Role of TMOF in gut phosphorylation

To find out if TMOF is a phosphatase inhibitor rather than ecto-protein kinase activator, different amounts of TMOF (0-100  $\mu\text{g}$ ) were incubated with alkaline phosphatase (1  $\mu\text{L}$ ; Sigma) in the presence of p-nitrophenyl phosphate (200  $\mu\text{g}$ ) in diethanolamine buffer pH 9.8 for 5-20 min. Absorbance was read at 405 nm using an ELISA reader at 5 min intervals. Controls were run without TMOF and in the presence of phosphatase inhibitor cocktail 1 and 2 (Sigma). No inhibition was observed when alkaline phosphatase was incubated alone with p-nitrophenyl phosphate (Table 1, control). In the presence of phosphatase inhibitors cocktails 1 and 2 (1  $\mu\text{L}$  each), the phosphatase activity was inhibited 4.5-fold (Table 1), however, increasing amounts of TMOF (10 to 100  $\mu\text{g}$ ) did not significantly decrease or increase the activity of alkaline phosphatase ( $p < 0.05$ , Table 1). Similar results were obtained when protein phosphatase-1 catalytic subunit (Sigma) was incubated with TMOF (results not shown).

**Table 1.** The effect of TMOF and phosphatase inhibitors on alkaline phosphatase

Alkaline phosphatase was incubated with p-Nitrophenyl phosphate:	N	Units $\pm$ S.E.M.
a. Alone	6	0.767 $\pm$ 0.05 <sup>ab</sup>
b. Phosphatase inhibitors	6	0.169 $\pm$ 0.04 <sup>bcd</sup>
c. TMOF 10 $\mu$ g	3	0.906 $\pm$ 0.05 <sup>ac</sup>
d. TMOF 50 $\mu$ g	3	0.729 $\pm$ 0.047 <sup>ad</sup>
f. TMOF 100 $\mu$ g	3	0.720 $\pm$ 0.132 <sup>ac</sup>

Alkaline phosphatase (1  $\mu$ L) was incubated with p-Nitrophenyl phosphate in the presence and absence of phosphatase inhibitor cocktails 1 and 2 (Sigma) or TMOF (10-100  $\mu$ g) at room temperature in diethanolamine buffer (200  $\mu$ L), 5 mM MgCl<sub>2</sub>, pH 9.8, for 5-10 min. Absorbance was read at 405 nm and enzyme activity expressed as units ( $\mu$ mole p-nitrophenyl phosphate/min) is the average of 3-6 determinations  $\pm$ S.E.M. <sup>a</sup>No significant difference by Student's t test  $p > 0.05$ , <sup>bcd</sup>Significant difference by Student's t test  $p < 0.05$ .

These results show that TMOF binding to its gut receptor activates an ecto-protein kinase that phosphorylates a membrane protein, presumably the TMOF gut receptor that is cleaved from the gut by a specific protease that resides on the gut's surface. The phosphorylation and the release of the protein from the gut probably activates the TMOF gut's receptor (directly or indirectly) in an analogous mechanism that is found for protease activated highly expressed G-protein coupled receptors in mammals platelets, on endothelial cells, myocytes and neurons. The signal transduction pathway that is activated by the binding of TMOF to its gut receptor is not known at this time.

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