ISJ 3: 137-145, 2006 ISSN 1824-307X

Research Report

Brush border membrane vesicles from dipteran midgut: a tool for studies on nutrient absorption

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Accepted December 20, 2006

Abstract

Brush border membrane vesicles (BBMV) from insects midgut can be successfully used to study several membrane phenomena, including nutrient absorption, ions permeability and insecticides mode of action. Midgut BBMV, purified from *Musca domestica* whole larvae, were used for the functional characterization of leucine transport. The amino acid uptake was accelerated in the presence of sodium or potassium and increased significantly when the extravesicular pH was 5.0, in agreement with the luminal pH *in vivo*. Radiolabelled leucine uptake was significantly reduced by an excess of cold leucine, histidine, serine and glycine, suggesting that the amino acid transporter is a broad scope carrier that does not recognize proline, glutamine and the dibasic amino acids lysine and arginine.

Midgut BBMV were also obtained from homogenization of *M. domestica* and *Bactrocera oleae* adults. The final preparations showed a high enrichment in the specific activity of the BBM marker enzymes aminopeptidase N and γ-glutamyl transpeptidase, and were poorly contaminated by basolateral membranes, as indicated by the low specific activities of their marker enzyme Na^+/K^+ ATPase. Electron microscopy of *B. oleae* BBM fraction showed the presence of closed vesicles. Similar SDS-PAGE patterns, with numerous distinct bands, were detected for both *B. oleae* and *M. domestica* BBMV.

Key words: dipteran midgut; brush border membrane vesicles; amino acid absorption; *Bactrocera oleae*; *Musca domestica*

Introduction

Amino acids play a key role in several physiological processes and for this reason intestinal amino acid absorption, a crucial step in nitrogen metabolism, affects the biological development of the whole organism. The functional and regulative properties of the amino acid transport systems expressed in the insect midgut epithelium has been extensively studied in lepidopteran larvae (Giordana *et al.*, 1989, 1998, 2002; Wolfersberger, 1996; Leonardi *et al*., 1998a, 2001a, b; Casartelli *et al*., 2001; Parenti *et al*. 2002) while little is known on intestinal amino acid absorption in other insect orders (Hong *et al*., 1995, 1997; Neal, 1996; Neal *et al*., 1996; Parenti *et al*., 1986, 2001).

Brush border membrane fragments form sealed vesicles spontaneously maintaining their correct orientation (Haase *et al.*, 1978), that allow to define the internal and external environments according to the experimental needs. The brush border membrane vesicles (BBMV) isolated from lepidopteran midgut epithelium have been successfully used for the functional characterization of amino acid transport proteins (Giordana *et al*., 1989, 1998; Wolfersberger, 1996; Leonardi *et al*., 1998a; Casartelli *et al*., 2001), to analyze *in vitro* the activity of the insecticide fenoxycarb (Leonardi *et al*. 1996, 1998b, 2001a) and, far more extensively, to study the mode of action of

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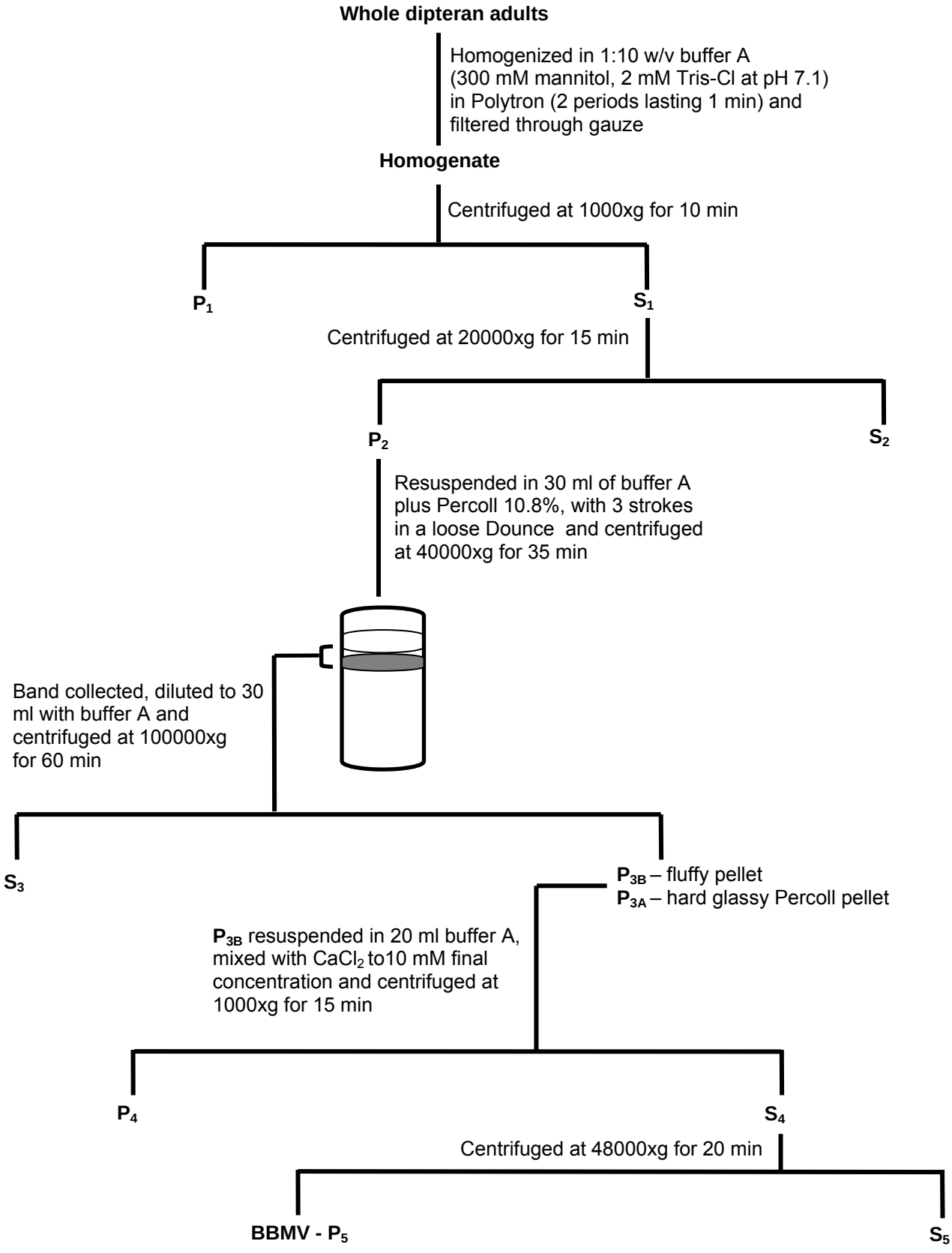

Fig. 1 Procedure for the preparation of BBMV from adults *M. domestica* and *B. oleae* (described in detail in Materials and Methods).

Cry1 toxins of *Bacillus thuringiensis* (Sacchi *et al*. 1986; Gill *et al.*, 1992; Giordana *et al*., 1993; Knowles, 1994; Leonardi *et al*., 1997; Bravo *et al.*, 2002). The preparation of purified BBMV from the midgut of insects of interest is therefore fundamental to investigate several membrane phenomena.

In the present paper we describe the preparation of purified midgut BBMV using whole *Musca domestica* larvae as a starting material. The vesicles were then used to describe the functional properties of amino acid uptake in this dipteran larva.

We also describe a method to obtain midgut BBMV from adults of *M. domestica* and *Bactrocera oleae*, preparations that can be used to study the physiological processes active at the apical membrane of midgut cells or the effect on the same membrane of insecticides potentially active against the adults of these two dipteran pests.

Materials and Methods

Materials

L-[4,5-^{3}H]Leucine (71 Ci/mmol) was purchased from Amersham Biosciences Europe, Italy. All the other reagents were supplied by Sigma-Aldrich s.r.l., Italy.

Experimental animals

M. domestica larvae, kindly provided by Dr. Marcello Verdinelli (Istituto di Ricerca sul Controllo Biologico dell'Ambiente (IRCOBA), CNR, Sassari), were immediately frozen and preserved in liquid nitrogen for few weeks.

Pupae of *M. domestica* and *B. oleae*, also provided by IRCOBA, were maintained at 25 ± 1 °C, 65-70 % R.H. and 12L:12D photoperiod. Immediately after eclosion, the adults were anaesthetized at 4 °C, then frozen in liquid nitrogen and there preserved for few weeks.

BBMV preparation from larvae of M. domestica

Larvae of *M. domestica*, removed from liquid nitrogen, were placed in 1:10 w/v of an ice-cold buffer composed of 100 mM mannitol, 10 mM Hepes-Tris at pH 7.1, 1 mM PMSF and homogenized on ice with Polytron (Kinematica CH-6010 KRIENS-LU) for 3 periods lasting 30 s at velocity 5. The homogenate was filtered through 2 gauze layers and BBMV were prepared by Ca^{++}-precipitation and differential centrifugation as reported by Giordana *et al*. (1982). The filtered homogenate (H) was added with $CaCl_2$ to a final concentration of 10 mM, the suspension was stirred on ice for 15 min and then centrifuged at 3000 x g for 15 min. The supernatant was decanted and kept on ice. The pellet was resuspended in half of the original volume of buffer (100 mM mannitol, 10 mM Hepes-Tris at pH 7.1) with the aid of a glass-teflon homogenizer (IKA-Labortechnick RE 16) with five strokes at 1500 rpm. The suspension was mixed with $CaCl_2$ to a final concentration of 10 mM, blended on ice for 15 min and then centrifuged at 3000 x g for 15 min. The second pellet was discarded. The first and the second supernatants were pooled and centrifuged at 48000 x g for 20 min. The supernatant was discarded and the pellet resuspended in the original volume of buffer with five strokes at 1500 rpm in the glass-teflon homogenizer. The suspension was centrifuged at 48000 x g for 20 min and the final pellet, containing the brush border membranes, was resuspended in a suitable amount of buffer by 10 passes through a 22 gauge needle.

Protein concentration was assessed with the Coomassie Brilliant Blue G-250 protein assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard.

BBMV preparation from adults of M. domestica and B. oleae

The procedure is schematically presented in Fig. 1. All the steps were performed at 4 °C. The frozen adults were placed in ice-cold buffer composed of 300 mM mannitol, 2 mM Tris-Cl at pH 7.1, in a 1:10 w/v proportion, and homogenized with a Polytron (Kinematica CH-6010 KRIENS-LU) for 2 periods lasting 1 min at velocity 5. The homogenate was filtered through 3 layers of gauze to remove large debris. The filtered homogenate (H) was centrifuged at 1000 x g for 10 min. The first pellet P_1 was discarded and the supernatant S_1 was centrifuged at 20000 x g for 15 min. The supernatant S_2 was discarded and the pellet P_2 resuspended in 30 ml of buffer containing 10.8 % of Percoll with 3 strokes in a loose Dounce homogenizer. The suspension was centrifuged at 40000 x g for 35 min with no brake. The plasma membranes were present as a band at the third upper portion of the continuous density gradient formed by Percoll. The membrane fraction was carefully removed with a Pasteur pipette, diluted to 30 ml with buffer and centrifuged at 100000 x g for 1 h.

The supernatant S_3 was carefully decanted and the fluffy membrane layer P_{3B}, separated from the glassy Percoll pellet P_{3A}, was resuspended in 20 ml of buffer. $CaCl_2$ was added to the suspension to a final concentration of 10 mM, the mixture was blended on ice for 15 min and then centrifuged at 1000 x g for 10 min. The pellet P_4 was discarded and the supernatant S_4 centrifuged at 48000 x g for 20 min. The final pellet P_5, containing the brush border membranes, was resuspended in a suitable volume of buffer by means of 10 passes through a 22 gauge needle.

Enzyme assays

Enzyme activities were measured in the filtered homogenate (H) and in BBMV. Aminopeptidase N (EC 3.4.11.2) was determined by measuring the release of *p*-nitroaniline from L-leucine-*p*-nitroanilide in 40 mM TrisHCl at pH 7.5 and γ-glutamyl transferase (E.C. 2.3.2.2) by the release of 5-amino-2-nitrobenzoate from L-γ-glutamyl-glycilglycine in 100 mM TrisHCl at pH 8. Incubations were carried out in a spectrophotometer (Ultrospec 3000 Pharmacia Biotech, Cambridge UK) with a thermostatic (25 °C) cuvette holder, in conditions in which activity was proportional to the protein concentration and time.

Table 1 Protein yield and activities of plasma membrane marker enzymes in homogenate and BBMV from *M. domestica* larvae

	Protein yield (mg protein/g animals)		
BBMV	0.31 ± 0.09 (5)		
	Enzyme activity (mU/mg protein)		
	aminopeptidase N	γ-glutamyl transferase	Na^+/K^+ ATPase
Homogenate	84.1 ± 3.0 (5)	2.0 ± 0.1 (3)	121 ± 21 (3)
BBMV	642 ± 119 (5)	20.6 ± 0.6 (3)	189 ± 8 (3)
Enrichment factor*	7.5 ± 1.2 (5)	10.3 ± 0.2 (3)	1.7 ± 0.4 (3)

Values are means ± SE In brackets are reported the number of independent preparations assayed
*Ratio of the enzyme specific activity in BBMV and in the homogenate

One unit of enzyme activity corresponds to the hydrolysis of 1 µmol of substrate/min.

Na^+/K^+-ATPase (E.C. 3.6.1.3.) activity was measured according to Quigley and Gotterer (1969).

Electrophoresis

Brush border membrane proteins were analyzed under denaturing conditions by SDS-PAGE (Laemmli, 1970) in a Mini Protean II Bio-Rad electrophoresis system. 4 % and 10 % acrylamide concentrations were used for stacking and running gels respectively. Samples were prepared by mixing 1:1 (v/v) H or BBMV with the loading buffer 2X (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromphenol blue, 0.125 M Tris-HCl at pH 6.8) (Sigma-Aldrich s.r.l., Italy). The mixture was incubated at 100 °C for 4 min before loading the samples. The bands were stained with Coomassie Blue R-250.

Transmission and scanning electron microscopy

BBMV were fixed for 30 min in 0.1 M cacodylate buffer, pH 7.2, containing 2 % glutaraldehyde. Samples were then washed in the same buffer and postfixed for 20 min with 1 % osmic acid in 0.1 M cacodylate buffer, pH 7.2. After a standard step of serial ethanol dehydratation, BBMV were pelletted and embedded in an Epon-Araldite 812 mixture. Sections were obtained with a Reichert Ultracut S ultratome (Leica, Wien, Austria). Thin sections were stained by uranyl acetate and lead citrate and observed with a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan).

Measurements of leucine uptake in BBMV from M. domestica larvae

Transport experiments were performed in triplicate at room temperature by rapid filtration (Hanozet *et al.*, 1980). To measure leucine uptake, 1 volume of the BBMV suspension was mixed to 4 volumes of the radiolabelled incubation medium, whose final concentration is reported in the legend of the Figures. Uptake was terminated by diluting the incubated mixture with 50 volumes of ice-cold stop solution (150 mM NaCl, 10 mM Hepes-Tris at pH 7.2). The suspension was then filtered through a prewetted mixed cellulose ester filter (0.45 µm pore size, Micro Filtration Systems, Dublin, CA), then counted for radioactivity in a scintillation spectrometer (Tri-Carb, Packard, model 1600 CA).

Results and Discussion

In the 1980's two methods were published to prepare BBMV from the midgut of lepidopteran larvae (Hanozet *et al.*, 1980; Giordana *et al.*, 1982; Wolfersberger e*t al.*, 1987). These procedures required that the midgut tissues were isolated from the larvae and were based on the calcium- or magnesium-precipitation of the membrane fragments obtained from the tissue homogenization, followed by differential centrifugations. Later, modifications of the original protocols were introduced to prepare midgut BBMV sufficiently purified starting directly from the entire larva of the Lepidoptera *Plutella xilostella* (MacIntosh *et al.*, 1994) or the Diptera *Aedes aegypti* (Abdul-Rauf and Ellar, 1999) and *Chironomus riparius* (Parenti *et al.*, 2000).

The use as a starting material of the entire animal instead of the isolated midguts reduces enormously the time necessary to obtain BBMV. We applied the Ca^{++}-precipitation method (Giordana *et al.,* 1982), conveniently modified, to the larval stage of the important pest *M. domestica*. A satisfying homogenization of all the larval tissues could be obtained only by using a Polytron homogenizer, and an adequately purified final preparation was achieved by adding a second cycle of Ca^{++}-precipitation and

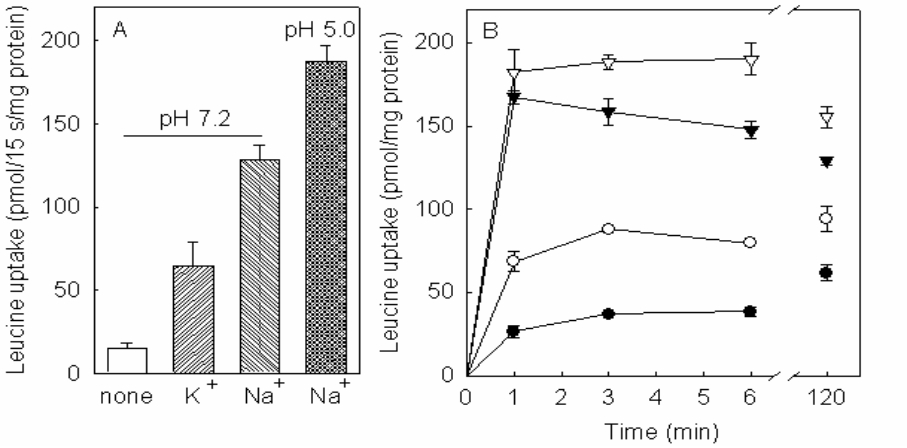

Fig. 2 Initial rates (panel A) and time course (panel B) of leucine uptake in BBMV from *M. domestica* larvae. BBMV, resuspended in 100 mM mannitol, 10 mM Hepes-Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 0.1 mM L-[4,5-^{3}H] Leucine 30 µCi/ml, 100 mM NaSCN (Na^+, ▼□) or KSCN (K^+, ○), or 200 mM sucrose (none, ●), 20 mM Hepes-Tris at pH 7.2 or 20 mM Mes-Tris at pH 5.0 (Na^+, □). Values are means ± SE of a typical experiment performed in triplicate. Initial rates (panel A) and time course (panel B) of leucine uptake in BBMV from *M. domestica* larvae. BBMV, resuspended in 100 mM mannitol, 10 mM Hepes-Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 0.1 mM L-[4,5-^{3}H] Leucine 30 µCi/ml, 100 mM NaSCN (Na^+, ▼□) or KSCN (K^+, ○), or 200 mM sucrose (none, ●), 20 mM Hepes-Tris at pH 7.2 or 20 mM Mes-Tris at pH 5.0 (Na^+, □). Values are means ± SE of a typical experiment performed in triplicate.

differential centrifugation. The purity was verified by measuring the specific activity in the homogenate and in the final pellet of aminopeptidase N and γ-glutamyl transferase, two enzymes exclusively localized in the apical membrane of midgut cells, and of Na^+/K^+ ATPase, the marker enzyme of the basal plasma membrane. The enrichment factor was calculated as the ratio between the enzyme specific activity in the final preparation and in the homogenate. The brush border marker enzymes were enriched sevenfold and tenfold, respectively, with a negligible enrichment of Na^+/K^+ ATPase (Table 1), so the final BBMV preparation was essentially free of contaminating basal membranes. Altogether, the enrichment factors of this study are consistent with previous results, since a tenfold enrichment of the BBM marker enzymes were obtained for BBMV prepared from whole *Aedes aegypti* larvae (Abdul-Rauf and Ellar, 1999), and enrichment factors varying between 3 and 5 were calculated for BBM purified from the isolated larval midguts of *M. domestica* as a starting material (Lemos and Terra, 1992; Jordao *et al.,* 1995).

We performed transport experiments in *M. domestica* BBMV to identify the main features of amino acid absorption in this insect. The time course of 0.1 mM leucine uptake into BBMV was measured in the absence of cations, or in the presence of an inwardly directed sodium- or potassium-gradient. If an amino acid is transported by a transport protein that also binds and translocates a cation, i.e. by a cotransporter, a transient accumulation of the amino acid in the intravesicular space should occur, due to the flow of the cation along its electrochemical gradient. Otherwise, the uptake is merely equilibrative and the equilibrium will be attained at different times, according to the amino acid transport rate. When the extravesicular pH was 7, leucine initial uptake rate at 15 s increased fourfold in the presence of K^+ and eightfold with Na^+ (Fig. 2, panel A). The time course of leucine uptake in the absence of cations was very slow and the equilibrium was not yet reached after 120 min (Fig. 2, panel B). In the presence of a K^+- or a Na^+-gradient, leucine uptake was accelerated but no transient accumulation of the amino acid was observed

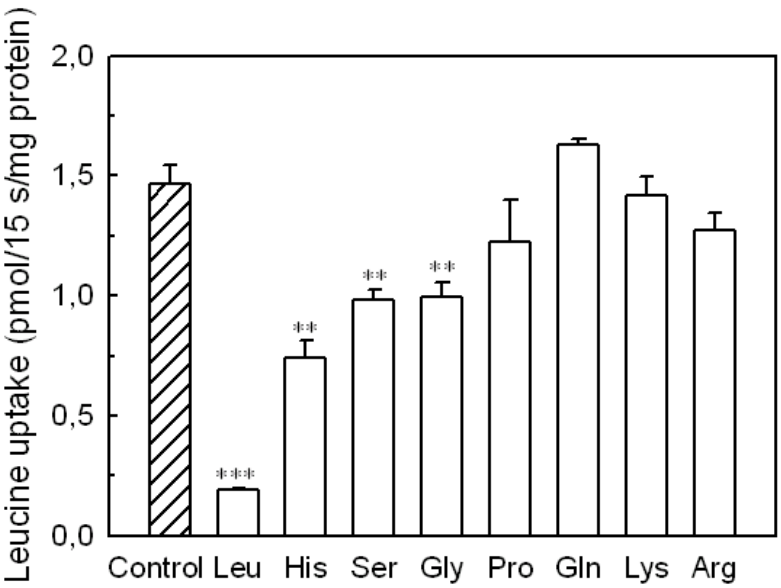

Fig. 3 Inhibition of radiolabelled leucine uptake by a large excess of the indicated amino acids in BBMV from *M. domestica* larvae. BBMV, resuspended in 100 mM mannitol, 10 mM Hepes-Tris at pH 7.2, were diluted 1:5 to obtain the following final composition: 0.4 µM L-[4,5-^{3}H]Leucine 30 µCi/ml, 100 mM NaSCN, 10 mM inhibitors or mannitol (control), 20 mM Hepes-Tris at pH 7.2. Values are means $\pm$ SE of a typical experiment performed in triplicate. The statistical analysis of the data was performed by Student's *t*-test. ** $p < 0.01$; *** $p < 0.001$.

(panel B). Therefore, Na^+ and K^+ are able to activate leucine transporter in *M. domestica* midgut, but no evidence is given that the protein is a cotransporter. In coleopteran larval midgut, the uptake of tyrosine and methionine, but not that of leucine, was also stimulated by Na^+ and K^+ (Hong *et al.*, 1995) and, in agreement with our results, no accumulation of the two amino acids was observed with a Na^+- or a K^+-gradient (Neal, 1996).

The Na^+-dependent leucine uptake increased markedly when the extravesicular pH was 5.0 (Fig. 2, panels A and B), suggesting that leucine transporter is adapted to the physiological environment of *M. domestica* larval midgut, where the lumen contents is weakly acidic in the anterior and posterior regions and highly acidic in the middle region (Terra, 1988; Dubreuil, 2004). Interestingly, leucine uptake in midgut BBMV of lepidopteran larvae is mediated by a transport system that is, in that case, strongly activated by an extravesicular alkaline pH, as tipically present in the luminal fluids of the larval midgut: the pH-gradient can be exploited to perform leucine intravesicular accumulation (Giordana *et al.*, 1998). Therefore, the ability to operate more efficiently in the presence of the physiological pH is a functional property in common between dipteran and lepidopteran leucine transport systems.

As expected for a carrier-mediated process, a 87 % reduction of radiolabelled leucine uptake was observed in the presence of a large excess of cold leucine (Fig. 3).

The transporter recognized other neutral amino acids, since a 56 %, 34 % and 37 % inhibition was observed with an excess of histidine, serine and glycine, respectively (Fig. 3). The percent inhibitions were calculated with respect to the control value after subtraction of the residual uptake in the presence of an excess of leucine. The uptake was not affected by proline, glutamine or by the dibasic amino acids lysine and arginine, suggesting the presence of other transporters for the intestinal absorption of these amino acids.

While protocols for the preparation of BBMV from whole larvae are now available (Abdul-Rauf and Ellar, 1999; Parenti *et al.*, 2000; this paper), no procedures are actually known to obtain BBMV directly from adult diptera, a severe limit to the investigation of the physiological processes occurring at the apical membrane of midgut cells and to the detection of new drugs potentially noxious to the brush border, that may be used as pesticides. We have, therefore, developed a new procedure based on the fractionation of the homogenate on a continuous density gradient of Percoll, followed by calcium-precipitation (Fig. 1).

A complete homogenization of *B. oleae* and *M. domestica* adults was obtained with the Polytron homogenizer. The brown-black color developed during homogenization remained in the pellet of the first low-speed centrifugation, that is discarded, so that no dark coloration was observed in the following steps of the procedure. The plasma membranes stratified as a distinct diffused band located at the third upper portion of the continuous density gradient formed by Percoll. The residual Percoll present in the collected fraction was later removed as a glassy pellet after the high speed centrifugation.

Table 2 Protein yield and activities of the plasmamembrane marker enzymes in homogenate and BBMV from *M. domestica* and *B. oleae* adults

	Protein yield (mg protein/g insects)	
	B. oleae	*M. domestica*
BBMV	0.03 ± 0.01 (3)	0.13 (2)

	Enzyme activity (mU/mg protein)		
B. oleae	aminopeptidase N	γ-glutamyl transferase	Na^+/K^+ ATPase
Homogenate	19.0 ± 0.04 (3)	2.0 ± 0.1 (3)	83 ± 7 (3)
BBMV	531 ± 18.2 (3)	58.3 ± 6.8 (3)	275 ± 24 (3)
Enrichment factor*	31.9 ± 9.6 (3)	28.8 ± 3.2 (3)	3.3 ± 0.2 (3)
M. domestica	aminopeptidase N	γ-glutamyl transferase	Na^+/K^+ ATPase
Homogenate	17.2 (2)	not detectable	380 ± 40 (2)
BBMV	65.0 (2)	52.2 (2)	not detectable
Enrichment factor*	3.8 (2)	-	-

Values are means ± SE In brackets are reported the number of independent preparations assayed
*Ratio of enzyme specific activity in BBMV and in the homogenate

As shown in Table 2, the final pellet obtained from *B. oleae* was enriched 30-fold in aminopeptidase N and γ-glutamyl transferase activities with a very low enrichment for Na^+/K^+ ATPase, a reliable indication that the protocol leads to a highly purified brush border membrane preparation. However, BBMV protein yield was extremely low (Table 2), a result more likely related to the fact that the intestinal epithelium represents a very small portion of the starting material rather than to the complexity of the procedure. The microphotograph of the final pellet (P_5) indicated the presence of closed vesicles and of some amorphous material (Fig. 4).

The analysis of the purity of the BBMV from *M. domestica* (Table 2) confirms that this final preparation is also fairly purified. The enrichment factor of aminopeptidase N was lower than that obtained for *B. oleae* but the final pellet was not contaminated by basal membranes, since no Na^+/K^+ ATPase activity was detectable. The enrichment in brush border membranes was also confirmed by the emergence in the BBMV pellet of a γ-glutamyl transferase activity, too diluted in the homogenate to be detected.

A characterization of *B. oleae* and *M. domestica* BBMV was performed by SDS-PAGE (Fig. 5). Numerous distinct bands were resolved by the gel. The patterns produced by the BBMV of the two species were very similar (lanes 3 and 5), while they

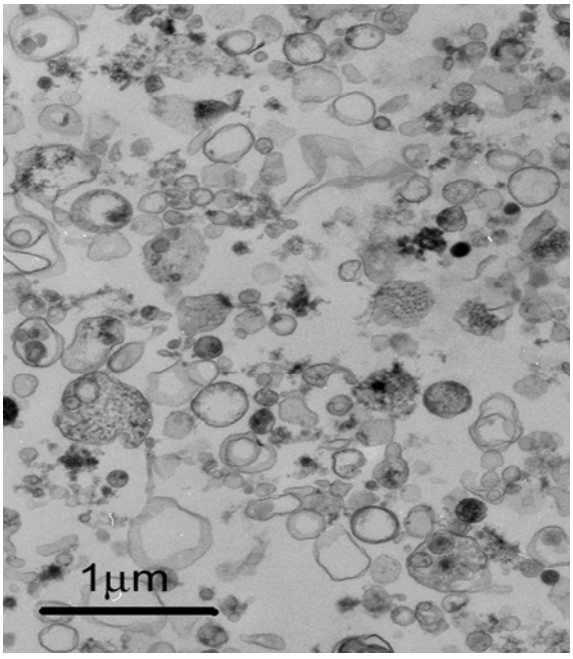

Fig. 4 Electron micrograph of the final membrane pellet from *B. oleae* whole adults.

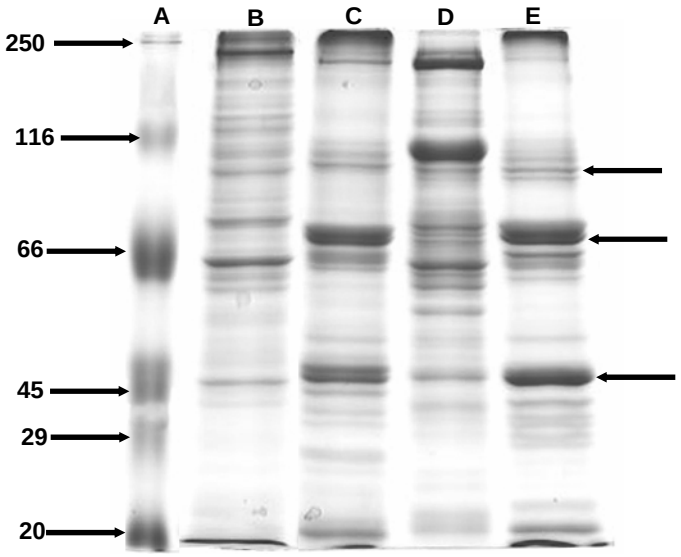

Fig. 5 Comparison of SDS-PAGE profile of BBMV prepared from adults of *B. oleae* (lane C) and *M. domestica* (lane E). Lane A) molecular weights (kDa); lane B) BBMV prepared from *Bombyx mori* larval midgut; lane D) homogenate from adults of *M. domestica*.

were evidently different from that obtained with midgut BBMV of a lepidopteran larva (lane 1) here presented for comparison. The apparent molecular masses of some major bands, present in the two dipteran BBMV and lacking in the homogenate (lane 4), correspond to a number of proteins involved in the architecture of microvilli cytoskeleton, such as actin (43 kDa), myosin I (110 kDa) and fimbrin (68 kDa).

In conclusion, our experimentation provides a midgut preparation from larvae of a pest insect, that can be used to characterize fundamental physiological processes such as amino acid absorption. The in depth knowledge of these processes may provide targets for new pesticides.

The novel preparation of midgut BBMV from dipteran whole adults, obtained for the first time, may contribute to solve the problem of the identification of the mechanism of action of insecticidal molecules acting on midgut brush border membrane in insects of small dimension.

Acknowledgements

We are grateful to Prof. M de Eguileor (Dipartimento di Biologia Strutturale e Funzionale, Università dell'Insubria, Italy) for the TEM image of BBMV pellet.

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