

# Copper-containing amine oxidase purified from *Lathyrus sativus* as a modulator of human neutrophil functions

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**Abstract.** Over the last few decades, copper-containing amine oxidase (Cu-AO) from vegetal sources, and belonging to the class of diamine oxidase, has been documented to exhibit beneficial effects in both *in vivo* and *ex vivo* animal models of inflammatory or allergic conditions, including asthma-like reaction and myocardial or intestinal ischemia-reperfusion injuries. The aim of the present study was to assess the potential of vegetal Cu-AO as an anti-inflammatory and an antiallergic agent and to clarify its antioxidant properties. In cell-free systems, the reactive oxygen species and reactive nitrogen species scavenging properties of Cu-AO that is purified from *Lathyrus sativus* were investigated. Its effect on the formyl-methionyl-leucyl-phenylalanine peptide (fMLP)-activated cellular functions of human neutrophils were subsequently analyzed. The obtained results demonstrated that Cu-AO is not a scavenger of superoxide or nitric oxide, and does not decompose hydrogen peroxide. However, it inhibits

the fMLP-dependent superoxide generation, elastase release and cell migration, and interferes with the process of calcium flux, supporting the idea that plant Cu-AO can interact with human neutrophils to modulate their inflammatory function. Therefore, the importance of these properties on the possible use of vegetal Cu-AO to control inflammatory conditions, particularly intestinal inflammation, is discussed in the current study.

## Introduction

An inflammatory response is the result of a complex and interconnected series of events of the immune system aimed at destroying foreign agents or altered cells, but inevitably leading to healthy tissue damage at the site of injury (1). In particular, activated neutrophils, which are key effectors of the innate immune responses, generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), proteolytic enzymes and neutrophil extracellular traps to eliminate pathogens and dying cells (2). However, these molecules can also interact with nucleic acids, proteins and lipids of the surrounding healthy tissues, causing changes in their structure and function with detrimental consequences for cellular homeostasis (3). The neutrophil-mediated activation of the immune response, as a causative or secondary factor, occurs in a broad range of pathologies, including post-ischemic reperfusion damage, neurodegeneration, obstructive pulmonary disease, obesity, diabetes and cancer (4). The excessive activated neutrophil recruitment also takes place in a variety of intestinal dysfunctions and inflammatory conditions, including intestinal ischemia and inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis (5,6). Therefore, there is a growing effort to understand the mechanisms associated with the action of neutrophils in inflammatory sites, and to identify novel factors that may modulate neutrophil behavior in order to develop therapeutic strategies to limit or neutralize their destructive action.

Serotonin, histamine, the putrescine diamine, the spermine and spermidine polyamines exert immunomodulatory, neuroactive and proliferative functions (7). Consequently, during the last few decades copper-containing amine oxidases (Cu-AOs) have been proposed as efficient anti-inflammatory and anti-allergic agents (8), due to their ability to metabolize the

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**Abbreviations:** AAP, aminoantipyrine; BSA, bovine serum albumin; BSAO, bovine serum Cu-AO; CRAC, calcium-release activated channels; Cu-AO, copper-containing amine oxidase; DAO, diamine oxidase; DCHBS, 3,5-dichloro-2-hydroxybenzenesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; fMLP, formyl-methionyl-leucyl-phenylalanine peptide; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; LSAO, Cu-AO purified from *Lathyrus sativus*; PBS, phosphate-buffered saline; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOCE, store-operated calcium entry; TG, thapsigargin; VAP-1, vascular adhesion protein 1

**Key words:** copper-containing amine oxidase, diamine oxidase, histaminase, neutrophils, oxidative stress, inflammation, inflammatory bowel disease

primary amine groups of these biogenic amines as follows (9):  $R-CH_2-NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$ . The beneficial effects of Cu-AOs from vegetal sources have been documented both in *in vivo* and *ex vivo* animal models of inflammatory or allergic conditions, including asthma-like reaction (10) and myocardial (11) or intestinal (12) ischemia-reperfusion injuries, in which the treatment with vegetal Cu-AO was demonstrated to promote the alleviation of these dysfunctions and the decrease of tissue histamine level, neutrophil infiltration and cellular oxidative damage. Also, the removal of histamine, which is an efficient substrate of vegetal Cu-AO (13) (for this reason the enzyme is also called histaminase, as well as diamine oxidase (DAO) for its ability to metabolize diamines), was associated with the anti-inflammatory function of this enzyme and its anti-allergic properties. Histamine, which is a major mast cell-derived mediator, is not only an efficient modulator of endothelial cell contraction and vascular permeability, but also a pro-inflammatory amine able to induce the expression of endothelial cell adhesion molecules, thus promoting the rolling and extravasation of leukocytes (14).

Cu-AO purified from bovine serum (BSAO) has been reported to exert a cardioprotective effect in isolated rat hearts exposed to electrolysis-generated ROS, and to have a scavenging capacity against ROS and their by-products generated by electrolysis in Krebs-Henseleit buffer (superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide and hypochlorous acid) (15), despite the fact that Cu-AOs generate hydrogen peroxide. It has been hypothesized that the beneficial effects of plant Cu-AO are associated with its scavenging capacity of ROS and RNS that are generated by activated neutrophils and the consequent reduction of tissue oxidative damage (8). However, direct evidence of the scavenging activity of vegetal Cu-AO is lacking.

With the aim to clarify the antioxidant and anti-inflammatory properties of vegetal Cu-AO and further define its potential use as a novel antiallergic and anti-inflammatory agent, the present study analyzed the scavenging properties of Cu-AO purified from *Lathyrus sativus* (LSAO) seedlings against ROS and RNS generated chemically in cell-free systems. Moreover, vascular adhesion protein 1 (VAP-1) is a peculiar Cu-AO of the plasma membrane of vascular endothelial and smooth muscle cells, and it is able to interact with specific determinants on the membrane of circulating leukocytes, promoting their extravasation to the site of the inflammation (16). Therefore, the ability of LSAO to directly affect neutrophil functions was studied *in vitro* by investigating the effects of the enzyme on some functional responses of isolated human neutrophils activated by formyl-methionyl-leucyl-phenylalanine peptide (fMLP). Fabaceae contain a high concentration of Cu-AO in the cellular periplasm (17). In particular, crude homogenates of *Lathyrus sativus* seedlings are a rich source of the enzyme (18), justifying the use of this plant.

## Materials and methods

**Materials.** Aminoantipyrine (AAP), bovine heart cytochrome *c*, bovine milk xanthine oxidase, bovine serum albumin (BSA), 4-bromo-calcium ionophore A23187, cytochalasin B, 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS), fMLP, glucose, horseradish peroxidase (HRP), hydrogen peroxide

( $H_2O_2$ ), N-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (MeO-Suc-Ala-Ala-Pro-Val-AMC), porcine pancreas elastase, sulphanilamide, sodium diethyldithiocarbamate (DDC), sodium nitroprusside, sodium nitrite, thapsigargin (TG), trypan blue and xanthine were purchased from Merck KGaA. Fura-2-acetoxymethyl ester (Fura-2AM) was purchased from Thermo Fisher Scientific, Inc., Polymorphprep™ solution from Sentinel Diagnostics and Superdex D200 FPLC column from GE Healthcare. Ultrafiltration devices with a cut-off of 30 kDa were obtained from Sartorius AG. QCM™ Chemotaxis 3  $\mu$ m 96-well cell migration assay system was from Merck KGaA. Other chemicals were analytical grade and were used without further purification.

**LSAO purification and characterization.** LSAO was prepared from *Lathyrus sativus* seedlings, according to a large-scale preparation method (19). Following purification, samples were dialyzed at 4°C overnight against 50 mM phosphate buffer (1:1,000; pH 7.4), concentrated on ultrafiltration devices and further purified by size exclusion chromatography on a Superdex D200 FPLC column, equilibrated and eluted with 50 mM phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min using a FPLC System (GE Healthcare). Aliquots with the highest AO-specific activity were pulled together, concentrated using ultrafiltration and stored at -20°C until use. The purified protein (10  $\mu$ g/lane) moved as a single band on 7.5% SDS-PAGE. LSAO preparations were characterized for protein content and enzymatic activity was measured in terms of  $H_2O_2$  generation from the oxidation of 3 mM putrescine, as previously reported (20). LSAO preparations with a specific activity of  $40 \pm 5$  U/mg protein were used. 1 U enzyme oxidizes 1  $\mu$ mol substrate/min.

Catalytically inactive LSAO samples were prepared upon the depletion of copper from the active site by DDC treatment (21). The residual copper content was measured as previously described (22).

**Neutrophil isolation.** Neutrophils were purified from heparinized human blood, as previously reported (23), freshly drawn from repeat healthy blood donors at the Immunohematology and Transfusion Medicine Unit at Policlinico Umberto I, Sapienza University of Rome (Rome, Italy) (~7 ml blood/donor). All repeat blood donors (23 women and 47 men; age 18-65 years) gave written informed consent approved by the Ethic Committee of Policlinico Umberto I, Sapienza University of Rome. Following separation in Polymorphprep™ density gradient medium by centrifugation at  $500 \times g$  for 30 min at 20°C, the cells were collected according to the manufacturer's protocol, suspended in PBS (pH 7.4), containing 140 mM NaCl, 2.7 mM KCl, 12 mM  $Na_2HPO_4$ , 1.5 mM  $KH_2PO_4$ , 5 mM glucose and 0.5 mM  $MgCl_2$  and stored on ice. Each preparation contained 90-98% neutrophils and was free of contaminating erythrocytes as detected using an inverted Axiovert 40 C microscope at a magnification of  $\times 100$  (Carl Zeiss AG). The neutrophils had a viability of >90% when kept in ice for up to 6 h after purification, as determined using the trypan blue exclusion test (24).

**Effect of LSAO on superoxide generated from the oxidation of xanthine by xanthine oxidase and from fMLP-activated**

*human neutrophils*. The effect of LSAO on superoxide, which was produced from the oxidation of xanthine by xanthine oxidase as previously reported (25), was measured for 20 min by adding 10  $\mu$ M LSAO enzyme to 50 mM phosphate buffer (pH 7.8) containing 10  $\mu$ M bovine heart cytochrome *c*, 0.1 mM EDTA, 50  $\mu$ M xanthine and bovine milk xanthine oxidase (sufficient to reduce cytochrome *c* at a rate of 0.025 absorbance unit per min in the absence of LSAO) in a spectrophotometric cuvette at 25°C.

To examine the effect of LSAO on the generation of superoxide by human neutrophils, a suspension of cells ( $2 \times 10^6$  cells/ml) was incubated at 37°C for 5 min in a spectrophotometric cuvette in PBS containing 60  $\mu$ M bovine heart cytochrome *c*, 1 mM  $\text{CaCl}_2$  and 1  $\mu$ g/ml cytochalasin B in the presence of 10  $\mu$ M LSAO. For neutrophil activation, 1  $\mu$ M fMLP was added to induce superoxide production (26).

In both cases, superoxide formation was evaluated by recording absorbance at 550 nm in a UV-1700 Shimadzu spectrophotometer (Shimadzu Corporation) to assess the change of absorbance associated with the superoxide-induced cytochrome *c* reduction. The concentration of reduced cytochrome *c* was calculated assuming an extinction coefficient of  $27,600 \text{ M}^{-1} \text{ cm}^{-1}$  (27).

*Scavenging effect of LSAO on  $\text{H}_2\text{O}_2$  and nitric oxide (NO)*. The effect of 10  $\mu$ M LSAO on  $\text{H}_2\text{O}_2$  was examined by incubating the enzyme at 25°C for 195 min in 50 mM phosphate buffer pH 7.0 containing 30  $\mu$ M hydrogen peroxide. The concentration of  $\text{H}_2\text{O}_2$  was estimated by adapting the previously reported method (20) to a microplate procedure, in which the presence of  $\text{H}_2\text{O}_2$  is detected by the formation of a pink adduct generated in the presence of AAP, DCHBS and HRP. At time intervals of 60 min, 110  $\mu$ l aliquots were withdrawn and added to 90  $\mu$ l of a solution containing 25 U/ml HRP, 4.5 mM AAP and 9 mM DCHBS on a 96 well-microplate. The microplate was incubated at room temperature for 15 min, and the optical density was then read at 515 nm using an Appliskan microplate reader (Thermo Fisher Scientific, Inc.). The  $\text{H}_2\text{O}_2$  concentrations were calculated using a standard curve obtained with stock solutions of  $\text{H}_2\text{O}_2$ , whose concentrations were estimated spectrophotometrically assuming a molar extinction coefficient of  $26,000 \text{ M}^{-1} \text{ cm}^{-1}$  (20).

The scavenging activity of LSAO on NO was evaluated in terms of the ability of the enzyme to decrease the rate of nitrite formation from NO generated in sodium nitroprusside solution, as previously reported (28). Briefly, sodium nitroprusside at a concentration of 50 mM was dissolved in 50 mM phosphate buffer (pH 7.4) continuously purged by  $\text{N}_2$ . Aliquots were then diluted ten times in 50 mM phosphate buffer (pH 7.4) containing 10  $\mu$ M LSAO and incubated at 25°C for 60, 150, 230 and 290 min. Aliquots were then withdrawn, added to an equal volume of Griess reagent (2% w/v sulphanilamide, 4% w/v  $\text{H}_3\text{PO}_4$ , 0.2% w/v naphthylethylenediamide) and the absorbance was read at 546 nm. NO concentration was expressed as nitrite using a standard curve prepared with sodium nitrite solutions at concentrations of 2.5, 5, 10, 25 and 50  $\mu$ M.

*Effect of LSAO on azurophilic degranulation in fMLP-activated human neutrophils*. Human neutrophils were incubated

for 5 min at a concentration of  $2 \times 10^6$  cells/ml at 37°C in PBS containing 1 mM  $\text{CaCl}_2$ , 1  $\mu$ g/ml cytochalasin B, different concentrations of LSAO (0.5, 1, 2.5, 5 and 10  $\mu$ M) and 40  $\mu$ M MeO-Suc-Ala-Ala-Pro-Val-AMC, a fluorogenic substrate of elastase. Cells were then exposed to 1  $\mu$ M fMLP and the concentration of released elastase was evaluated fluorimetrically on a Jasco-750 spectrofluorometer (Jasco Inc.) in terms of its catalytic activity by recording the fluorescence developed from the proteolysis of its substrate MeO-Suc-Ala-Ala-Pro-Val-AMC, as previously reported (29). To verify that LSAO did not inhibit the elastase catalytic activity, LSAO was incubated at 37°C with purified porcine elastase in PBS containing 1 mM  $\text{CaCl}_2$  and 1  $\mu$ g/ml cytochalasin B. The elastase activity was then recorded following the addition of 40  $\mu$ M MeO-Suc-Ala-Ala-Pro-Val-AMC as aforementioned.

*Effect of LSAO on cellular migration in fMLP-activated human neutrophils*. The effect of LSAO on neutrophil migration was assessed using the QCM™ Chemotaxis 3- $\mu$ m 96-well cell migration kit. Neutrophil suspension (100  $\mu$ l;  $1 \times 10^6$  cells/ml) in PBS containing 1 mM  $\text{CaCl}_2$  were added to the upper migration chamber of the provided microplate device; 150  $\mu$ l PBS aliquots were placed in the lower microplate chamber, and 10 nM fMLP and/or LSAO (1 and 10  $\mu$ M) were added. To avoid uncontrolled cell adhesion to the plates, PBS was supplemented with BSA at a final concentration of 1 mg/ml. The microplate was then kept in an incubator in a humidified atmosphere of 5%  $\text{CO}_2$  for 1 h at 37°C to allow the migration of neutrophils from the upper to the lower chamber. Following incubation, the upper migration chamber was removed, the wells of the lower chamber were treated for 15 min at room temperature with the lysis buffer and the dye solution according to the manufacturer's protocol, and the fluorescence of each sample, due to the binding of the dye to DNA, was read at a 535 nm emission upon excitation at 485 nm using an Appliskan microplate reader (Thermo Fisher Scientific, Inc.). The number of migrated cells was estimated using a standard curve obtained by adding from  $0.2 \times 10^5$  to  $1.0 \times 10^5$  total cells per well.

*Effect of LSAO on calcium flux in fMLP-activated human neutrophils*. Human neutrophils at a final concentration of  $10^7$  cells/ml were incubated with 5  $\mu$ M Fura-2AM, a specific calcium probe, for 30 min at 37°C in PBS. Cells were then washed, suspended in PBS at a final concentration of  $2 \times 10^7$  cells/ml and kept on ice to be used within the next 2-4 h. Neutrophil suspensions were diluted ten times in PBS buffer in a quartz cuvette, incubated at 37°C for 5 min and then exposed to different agents (1 mM  $\text{CaCl}_2$ ; 0.2 or 2 mM EGTA; 1  $\mu$ M fMLP; 10  $\mu$ M LSAO or BSA; 8 nM TG or 1.6  $\mu$ M 4-Bromo A23187). Fluorescence emission was measured at 485 nm upon excitation at 340 nm with a Jasco-750 spectrofluorometer (Jasco Inc.). The cytosolic free calcium concentration was calculated as previously described (30).

*Statistical analysis*. Data are expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed using one-way ANOVA with Tuckey's post hoc test for comparisons involving

three or more groups. GraphPad Prism® version 4.00 for Windows (GraphPad Software, Inc.) was used to perform statistical analysis.  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of LSAO on superoxide,  $H_2O_2$  and nitric oxide (NO).** Cu-AO prepared from *Lathyrus sativus* seedlings, at concentrations of  $10 \mu M$ , did not affect the rate of bovine heart cytochrome *c* reduction by superoxide generated from the oxidation of xanthine by xanthine oxidase (Fig. 1A). However, it slowed down the rate of cytochrome *c* reduction added to human neutrophils exposed to  $1 \mu M$  fMLP (Fig. 1B), indicating that the protein is not a scavenger of superoxide, but it is able to interfere with the fMLP-activated cellular mechanisms of superoxide generation.

LSAO did not interact with  $H_2O_2$  either, with no decrease of hydrogen peroxide concentration in solutions incubated with LSAO for 60, 120 and 195 min (Fig. 2A). Moreover, similar amounts of nitrite were generated from solutions of 5 mM nitroprusside in the absence or presence of  $10 \mu M$  LSAO, indicating that the enzyme did not scavenge NO (Fig. 2B).

### Effect of LSAO on fMLP-activated human neutrophil functional responses

**Azurophilic degranulation.** LSAO was indicated to markedly reduce the fMLP-induced azurophilic degranulation in human neutrophils as evaluated by elastase release (Fig. 3A). The activity of elastase released in neutrophil suspensions exposed to fMLP was lower in the presence of LSAO than in its absence, whereas in cell-free control experiments LSAO incubated with purified porcine elastase did not affect its proteolytic activity (data not shown). The effect of LSAO on the elastase released from neutrophils activated by fMLP was concentration-dependent with an  $IC_{50}$  of  $1.2 \pm 0.2 \mu M$  (Fig. 3A). Under the same experimental conditions, degranulation was not affected by  $10 \mu M$  BSA (data not shown), suggesting that the inhibition was LSAO-specific. The effect of the LSAO was not dependent on its catalytic activity. LSAO, inactivated by treatment with DDC, which removes copper from the active site of the enzyme, exhibited a similar effect as that of the catalytically active form (data not shown). In DDC-treated LSAO, the residual copper was  $<5\%$  of the original content, and the residual LSAO enzymatic activity was  $<3\%$ .

**Cellular migration.** Cu-AO from *Lathyrus sativus* was also indicated to interfere in a concentration-dependent manner with neutrophil migration. In the absence of fMLP, LSAO promoted a slight, but not statistically significant, migration of neutrophils when added at concentrations of 1 and  $10 \mu M$ . However, when neutrophils were activated by  $10 \text{ nM}$  fMLP,  $10 \mu M$  LSAO inhibited cell migration (Fig. 3B). Similar effects were observed with DDC-inhibited LSAO (data not shown). Furthermore, LSAO was not toxic under these experimental conditions. In fact, human neutrophils had a  $>90\%$  viability when incubated for 1 h in PBS containing 1 mM  $CaCl_2$  with or without 1 mg/ml BSA, either in the absence or in the presence of  $10 \mu M$  LSAO.

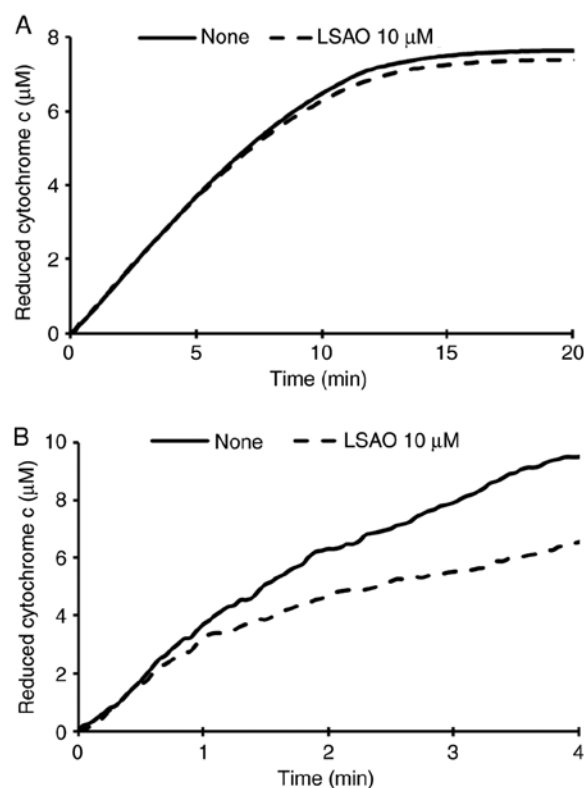


Figure 1. Effect of LSAO on superoxide. (A) Superoxide generation from the oxidation of xanthine by xanthine oxidase in the absence (continuous line) and presence (dashed line) of  $10 \mu M$  LSAO, expressed as reduced cytochrome *c*. One experiment representative of  $n=3$ . (B) Superoxide generation from fMLP-activated human neutrophils in the absence (continuous line) and presence (dashed line) of  $10 \mu M$  LSAO. One experiment representative of  $n=3$ . LSAO, Cu-AO-purified from *Lathyrus sativus*; fMLP, formyl-methionyl-leucyl-phenylalanine peptide.

**Calcium flux.** Exposure of human neutrophils to LSAO altered the calcium cellular homeostasis. In the presence of  $10 \mu M$  LSAO, the entry of calcium following the addition of 1 mM  $CaCl_2$  was lower compared with the absence of the enzyme (Fig. 4A), indicating that LSAO can interact with the neutrophil membrane systems responsible for calcium influx from the extracellular space. In addition, the enzyme inhibited  $\sim 50\%$  of the increase in the intracellular calcium level promoted by the exposure to  $1 \mu M$  fMLP, demonstrating that LSAO also interfered with the fMLP-dependent activation of calcium mobility. BSA ( $10 \mu M$ ) was not observed to affect cellular calcium mobility (Fig. 4A).

In neutrophils treated with 1 mM  $CaCl_2$  (to load the intracellular calcium reserves) and then exposed to 2 mM EGTA (to chelate the extracellular calcium, and detect the calcium efflux from the internal stores to the cytosol), the addition of  $10 \mu M$  LSAO caused a small, transient increase of the cytosolic calcium level (Fig. 4B). This behavior showed that LSAO may serve a role in the cell systems, promoting calcium mobility from the internal stores. Moreover, LSAO treatment affected the cytosolic calcium level increase when the cells were subsequently activated by  $1 \mu M$  fMLP (Fig. 4B). This indicated that LSAO also interfered with the fMLP-mediated release of calcium from the intracellular stores. In line with this evidence, following the addition of  $1.6 \mu M$  4-Bromo A23187 (a  $Ca^{2+}$  ionophore used in the

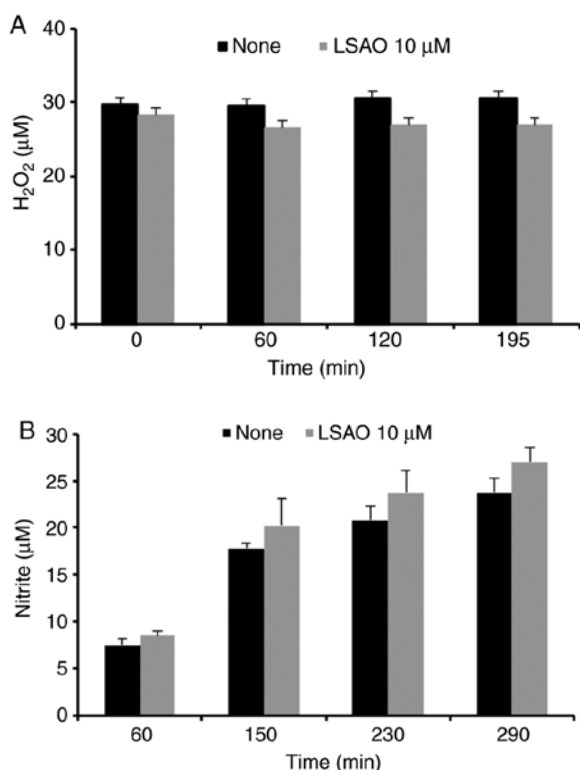


Figure 2. Effect of LSAO on H<sub>2</sub>O<sub>2</sub> or NO. (A) H<sub>2</sub>O<sub>2</sub> concentration in solutions not containing (black) or containing 10 μM LSAO (grey). Mean ± SD of n=4 experiments. For each time point the values of samples in the presence of LSAO were not statistically different from the values of samples in its absence. (B) NO generation, expressed as nitrite formation in solutions of nitroprusside not containing (black) or containing 10 μM LSAO (grey). Mean ± SD of n=3 experiments. For each time point the values of samples with LSAO are not statistically different from the values of samples in the absence of LSAO. LSAO, Cu-AO purified from *Lathyrus sativus*; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; NO, nitric oxide; SD, standard deviation; Cu-AO, copper-containing amine oxidase.

presence of EGTA to evaluate the concentration of calcium in the internal stores), the internal stores were indicated to be almost completely empty with a slightly higher residual calcium level in samples treated with LSAO (Fig. 4B). This confirmed that, in the presence of LSAO the fMLP-dependent release of calcium from internal stores was impaired. The exposure of neutrophils to 10 μM BSA did not affect cellular calcium mobility (Fig. 4B), suggesting again that the effect of LSAO was protein-specific.

The effect of LSAO on calcium influx in the absence of fMLP was confirmed in neutrophils treated with 8 nM TG (Fig. 4C), which inhibits the endoplasmic reticulum Ca<sup>2+</sup>-ATPase with consequent emptying of the intracellular calcium reserves and activation of the store-operated calcium entry (SOCE) (31). The SOCE is the major mechanism of calcium influx into neutrophils, through which the calcium-filling level in the endoplasmic reticulum regulates the entry of calcium from the extracellular space to the cytosol (32). In human neutrophils treated with TG, the presence of 10 μM LSAO inhibited the increase of the intracellular calcium level by ~40% when 1 mM CaCl<sub>2</sub> was added (Fig. 4C). This suggests a possible interaction of LSAO with the plasma membrane systems responsible for calcium entry, such as the calcium-release activated channels (CRAC) promoted by SOCE (33).

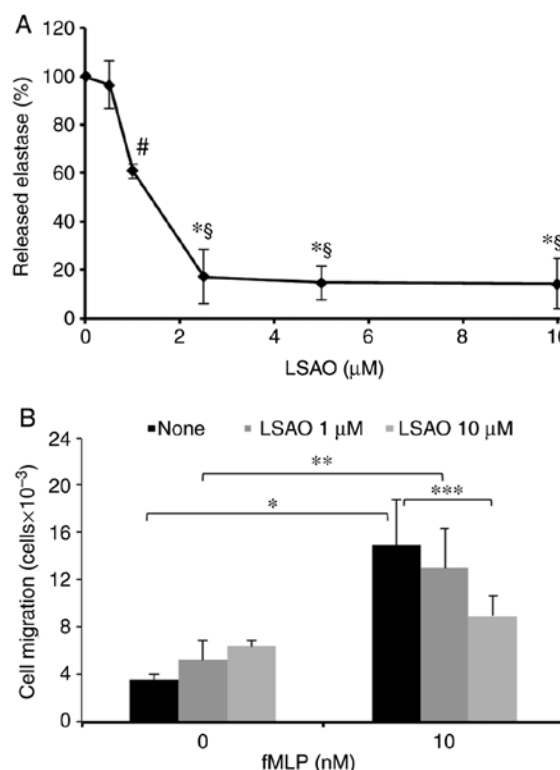


Figure 3. Effect of LSAO on elastase release and cellular migration in fMLP-activated human neutrophils. (A) Dose effect of LSAO on elastase release from neutrophils. Mean ± SD of n=3 experiments. Values are statistically different (\*P<0.0005 and \*P<0.0001 vs. control without LSAO; §P<0.0002 vs. LSAO 1 μM). (B) Migration of human neutrophils in the absence (black) or presence of 1 μM (grey) or 10 μM (light grey) LSAO. Mean ± SD of n=4 experiments. Values are statistically different (\*P<0.0001, \*\*P<0.0005 and \*\*\*P<0.005). LSAO, Cu-AO purified from *Lathyrus sativus*; fMLP, formyl-methionyl-leucyl-phenylalanine peptide; SD, standard deviation.

## Discussion

Over the last few decades, the administration of vegetal Cu-AO has been proposed as a potential antioxidant, anti-allergic and anti-inflammatory strategy, due to its beneficial effects in different animal model systems (8). This approach was also supported by its well-known ability to metabolize histamine (13) and by the capability of BSAO to scavenge electrolysis-induced ROS in Krebs-Henseleit buffer (15).

The current study into the properties of vegetal Cu-AO was extended to include an exploration of the ROS and RNS scavenging properties of the enzyme purified from *Lathyrus sativus* seedlings. Moreover, the effect of LSAO on neutrophil functions was analyzed. An altered activation of these cells is involved in the pathogenesis of different autoimmune and inflammatory conditions (i.e., Crohn's disease, ulcerative colitis, rheumatoid arthritis and systemic lupus erythematosus) (4). Therefore, the regulation of these functions is of therapeutic interest.

The obtained data indicated that Cu-AO purified from *Lathyrus sativus* seedlings does not have intrinsic scavenging properties on superoxide and hydrogen peroxide (Figs. 1A and 2A). This suggests that the previously reported scavenging capability of BSAO against ROS and their by-products, generated by electrolysis in Krebs-Henseleit



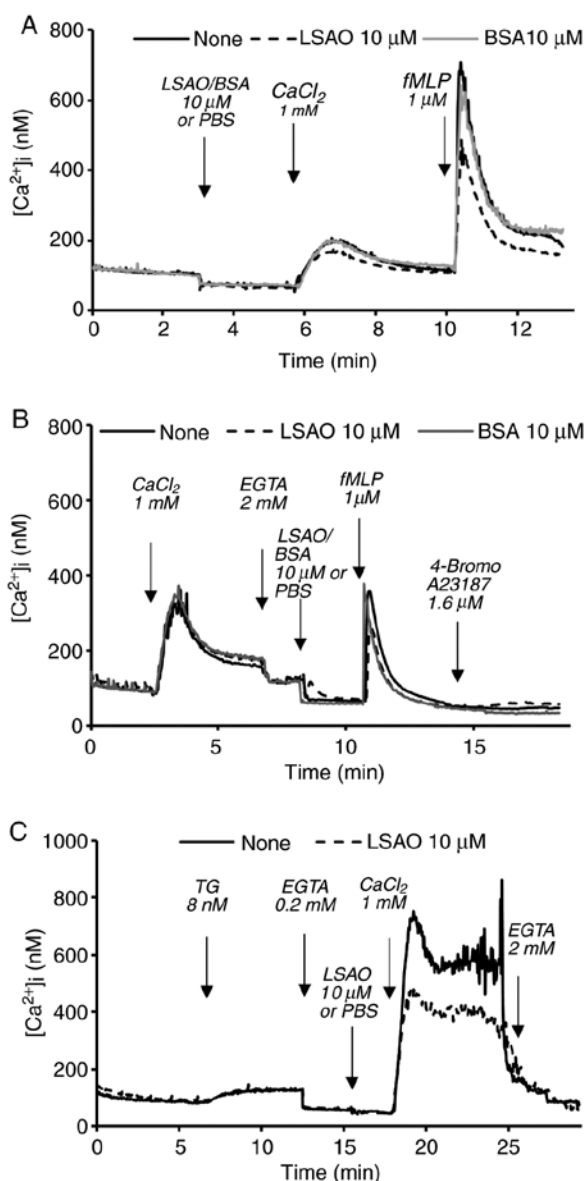


Figure 4. Effect of LSAO on: (A) Extracellular calcium entry into  $Ca^{2+}$ -depleted neutrophils and transient increase of  $[Ca^{2+}]_i$  induced by fMLP addition. One experiment representative of  $n=3$ . (B) Intracellular calcium release from internal stores triggered by fMLP. One experiment representative of  $n=3$ . (C) Store-operated calcium entry promoted by thapsigargin. One experiment representative of  $n=3$ . Different agents were added, as indicated by the arrows. Cells in the absence (continuous black line) or presence of 10  $\mu$ M LSAO (dashed black line) or 10  $\mu$ M BSA (continuous grey line). LSAO, Cu-AO purified from *Lathyrus sativus*; fMLP, formyl-methionyl-leucyl-phenylalanine peptide; BSA, bovine serum albumin;  $CaCl_2$ , calcium chloride; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; PBS, phosphate-buffered saline; TG, thapsigargin.

buffer (15) might be due to the interaction of this protein with reactive species other than superoxide and hydrogen peroxide, including hydroxyl radical, hypochlorous acid or singlet oxygen. Furthermore, the different behaviors of LSAO and BSAO might be due to different structural features. The primary structure of LSAO is not known; however, a sequence homology of ~23% has been reported between Cu-AO purified from pea and lentil seedlings with BSAO (34). In addition, LSAO is not a scavenger of NO (Fig. 2B). This is consistent with previous data showing that, in isolated hearts from guinea

pigs during anaphylaxis, pea-seedling Cu-AO promoted the release of NO, which would not be observed if the enzyme was a scavenger of NO (35).

Despite the lack of ROS and RNS scavenging activity, LSAO exerted an antioxidant effect by inhibiting the generation of superoxide in human neutrophils stimulated by fMLP (Fig. 1B). This effect might be due to a possible competition of the enzyme with fMLP interfering with its binding to FPR1, a G protein-coupled receptor constitutively expressed on the surface of neutrophils (36). Consequently, the activation of the cellular signaling pathways responsible for the assembly of the NADPH-oxidase complex may be reduced. However, a direct interaction of LSAO with the subunits gp91-phox and p22 of the NADPH oxidase complex located on the cellular membrane cannot be excluded, leading to an impaired complex assembly.

The binding of fMLP to FPR1 also regulates the exocytosis of the intracellular granules and vesicles, chemotactic migration and calcium release from endoplasmic reticulum (37,38). The exposure of neutrophils to fMLP in the presence of LSAO results in the inhibition of elastase release, cell migration (Fig. 3A and B) and calcium mobility (Fig. 4A and B), confirming that the vegetal enzyme can affect the interaction of fMLP with its receptor.

However, the ability of LSAO to affect cellular migration (Fig. 3B) and calcium mobility (Fig. 4B and C), in the absence of fMLP, suggests that in addition to FPR1, the vegetal enzyme can also directly interact with other components of the plasma membrane, contributing to the regulation of the neutrophil functions. In particular, the inhibition of calcium entry by LSAO in neutrophils treated with TG (Fig. 4C) suggests that vegetal DAO can directly interact with components of the CRAC channels responsible of calcium transport (i.e., stromal interaction molecule, calcium release-activated calcium modulator 1 and transient receptor potential channels) (33). Since fMLP-dependent signaling regulates neutrophil generation of superoxide, elastase release and chemotactic migration via cytosolic calcium increase (36), the capacity of LSAO to interfere with the CRAC-dependent calcium entry could also explain the inhibitory effect of the enzyme on the activation of neutrophils by fMLP.

The ability of LSAO to interact with neutrophil proteins is consistent with the capacity of free BSAO to interact with human hemoglobin (39) or that of gold-complexed BSAO to bind protein clusters on the surface of cultured human hepatocytes (40). In the latter case, the binding of the enzyme to the cellular membrane proteins was partially inhibited by N-acetyl-galactosamine, N-acetyl-glucosamine and mannose, suggesting that the BSAO carbohydrate chains are also important for protein-protein interaction. Although the carbohydrate moiety of LSAO has not been characterized, it is well known that pea and lentil Cu-AO are glycoproteins (41); therefore, it is possible that LSAO can associate with the proteins of the human neutrophil membrane via its glycosidic moiety.

In addition, BSAO has been documented to generate  $H_2O_2$  when incubated with polylysine, lysozyme or ribonuclease A (42), suggesting its ability to modify target proteins by the oxidative deamination of their lysines to the corresponding aldehydes. In addition, the vascular adhesion protein, VAP-1, of the endothelial cells, has been reported to hook

granulocytes, monocytes and lymphocytes and contribute to their extravasation through the formation of a covalent, but transient, Schiff base between its 2,4,5-trihydroxyphenylalanine cofactor and lysines of Siglec-9 and Siglec-10 proteins present on the surface of these white cells (43). However, under our experimental conditions, a modification of the neutrophil membrane proteins upon deamination of their amine groups by the LSAO enzymatic activity may be ruled out since the catalytically inactive LSAO was able to inhibit neutrophil functions, such as the release of elastase and cell migration.

In conclusion, the results of the current study indicated that LSAO up to 10  $\mu$ M concentration, reported to protect the human intestinal Caco-2 cell line from histamine-induced damage (20), is able to interfere with the activation of human neutrophils. This property, together with the ability to catabolyze histamine, confirms the anti-inflammatory effect of this enzyme and supports the use of vegetal Cu-AO for the treatment of inflammatory conditions; in particular enteric dysfunctions, including Crohn's disease and ulcerative colitis, where the transmigration of neutrophils into the intestinal lumen triggers drastic mucosal injury (44). In recent years, there has been increasing interest in the oral administration of vegetal Cu-AO as a novel complementary therapeutic strategy for the alleviation of food allergies and IBD symptoms (8). Vegetal Cu-AO has already been formulated for oral administration as monolithic tablets with carboxymethyl-starch (45). This is a pH-responsive excipient, which is protonated in gastric acidity, forming an external compact layer, and is then swollen and dissolved in neutral intestinal media where it favors the delivery of catalytically active enzymes. Furthermore, the effect of Cu-AO from *Lathyrus sativus*, which is rectally administered, in an *in vivo* murine intestinal inflammation model is currently under investigation (manuscript in preparation).

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

PP purified, modified and characterized the catalytic activity of LSAO, as well as its scavenging properties on ROS and RNS. PP also contributed to the isolation of neutrophils from whole human blood and to the analysis of calcium mobility,

performed the assay of cellular migration, collected and analyzed the data and contributed to manuscript editing. EC isolated neutrophils from whole human blood, designed and performed the study on calcium mobility and elastase release, analyzed the data and contributed to manuscript editing. MAM contributed to the concept of the intestinal administration of Cu-AO, the acquisition of funding and manuscript editing. LM purified, modified LSAO and characterized its catalytic activity, as well as its scavenging properties on ROS and RNS. LM also contributed to the analysis of calcium mobility, performed the assay of cellular migration, conceived and designed the current study, contributed to the acquisition of funding, collected, analyzed the data and wrote the original draft of the manuscript. All authors read and approved the manuscript.

### Ethics approval and consent to participate

Healthy blood donors gave written informed consent approved by the Ethics Committee of Policlinico Umberto I, Sapienza University of Rome (Rome, Italy).

### Patient consent for publication

Healthy blood donors gave written informed consent for publication.

### Competing interests

The authors declare that they have no competing interests.

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