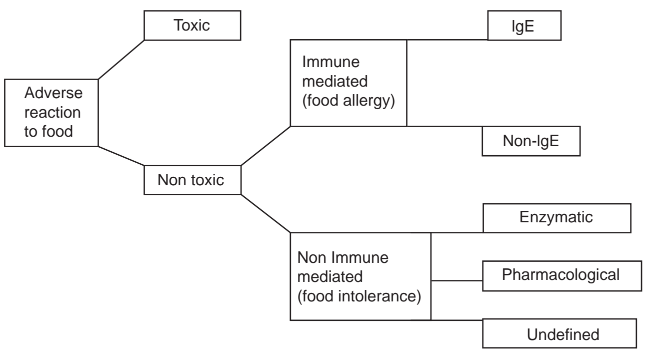

Figure 1 Adverse reactions of organism to food, EAACI classification.
Modified from 1.

permeability, increases mucosal secretion, causes tachycardia and arrhythmias, influences blood pressure, stimulates secretion of gastric juices and irritates nociceptive nerve fibres. Other important processes in which histamine is involved include neurotransmission, immunomodulation (enhanced chemotaxis of eosinophils and neutrophils, production of prostaglandins and thromboxane B, suppressed synthesis of lymphokines, etc.), haemopoiesis, wound healing, intestinal ischaemia, day-night rhythm, the regulation of histamine- and polyamine-induced cell proliferation and angiogenesis in tumour models.[3,4] Pleiotropic effects of histamine are mediated by its bond to membrane receptors of different cell types. Presently, there are four subtypes of histamine receptors described: histamine receptor 1 (H_1R), histamine receptor 2 (H_2R), histamine receptor 3 (H_3R) and histamine receptor 4 (H_4R). All these receptors belong to a family of receptors coupled with G-proteins. They are heptahelical transmembrane molecules, which act as transducers of extracellular signals via G-protein and intracellular system of second messengers.[5]

Endogenous sources of histamine in organism

Histamine originates in decarboxylation of amino acid histidine mediated by enzyme L-histidine decarboxylase, which contains pyridoxal phosphate (vitamin B6).[6] The name histamine comes from Greek *histos* – tissue, because it is present in many tissues of organism. It was isolated from liver and lung samples in 1927 by Best et al.[7] Classical sources of histamine in the organism are gastric enterochromaffin cells, histaminergic neurons, mast cells and basophils, which store histamine in intracellular vesicles, from where it is released upon stimulation. It is known that degranulation of mast cells and histamine release is a result of bonding of specific antigen to FcεRI receptor, which can be inhibited by luteolin (flavonoid with antioxidant properties).[60] Activation of mast cells can also occur in non-immune stimuli, such as neuropeptides (substance P), parts of complement system (e.g. C3a and C5a), cytokines (IL-1, IL-3, IL-8, and GM-CSF), platelet activating factor (PAF), hyperosmolarity, lipoproteins, adenosine, superoxidases and hypoxia. Many chemical and physical factors can be responsible for histamine release as well, for example extreme temperatures, trauma, vibrations or alcohol and some certain types of food and medication.[8] Mast cell activation plays a crucial role in the pathogenesis of many diseases – not only allergic, but autoimmune as well, such as rheumatoid arthritis.[61] Ability of de novo synthesis of histamine is also present in other cell types, e.g. platelets, monocytes/macrophages, dendritic cells, neutrophils and lymphocytes.[9]

Exogenous sources of histamine

Apart from endogenous production, histamine is introduced to the organism from exogenous sources by ingestion of some types of food, where histamine is naturally present in a high concentration. Histamine in exogenous sources can be synthesised by microbial decarboxylation of histidine by different fermenting bacteria, including natural human flora in the gut. Some bacteria are able to decarboxylate histidine in temperatures around +4 °C. To prevent histamine contamination of food the cooling is insufficient, freezing and early liquidation of viable bacteria is necessary. Due to thermostability, histamine which is present in food is almost irremovable. Some types of food contain naturally high amount of histamine (cocoa, spinach, tomatoes, ...). A high content of histamine is present in foods which originate by fermentation, either spontaneous or targeted (fermentation of alcoholic beverages – beer, wine; fermented vegetables, cheeses, meat, soy, yoghurt, ...). It is also important not to forget bacterial contamination of food when stored improperly.[10] The ability to produce histamine is present in Gram-positive, as well as Gram-negative bacteria. Many Gram-negative bacteria with this ability are common contaminants of food. From fish, ingestion of which caused histamine intoxication, the strains of *Hafnia alvei*, *Morganella morganii*, *Klebsiella pneumoniae*, *Morganella psychrotolerans*, *Photobacterium phosphoreum* and *Photobacterium psychrotolerans* were isolated. In fermented foods, the strains of *Oenococcus oeni*, *Pediococcus parvalus*, *Pediococcus damnosus*, *Tetragenococcus species*, *Leuconostoc species*, *Lactobacillus saerimneri 30a*, *Lactobacillus hilgardii*, *Lactobacillus buchnerii* and *Lactobacillus curvatus* are responsible for histamine production. Furthermore, it was discovered that for the contamination of ingredients in manufacturing process of wine and histamine production the strains of *Lactobacillus parabuchneri*, or *Lactobacillus rossiae* are responsible.[11] Enzymatic activity of histidine decarboxylase can last even after bacterial autolysis.[12]

Histamine degradation

Based on localisation, histamine can be inactivated by two processes – oxidative deamination of primary aminogroup to imidazolacetaldehyde, catalysed by enzyme diaminooxidase (DAO, histaminase)[13] or methylation of imidazole core to N4-methylhistamine catalysed by enzyme histamine N-methyltransferase (HNMT).[14] For proper function of DAO enzyme its cofactors are important – vitamins B6 and C and copper. DAO protein, stored in vesicular structures, bonds to plasma membrane of cells and is released in circulation after stimulation and is responsible for degradation of extracellu-

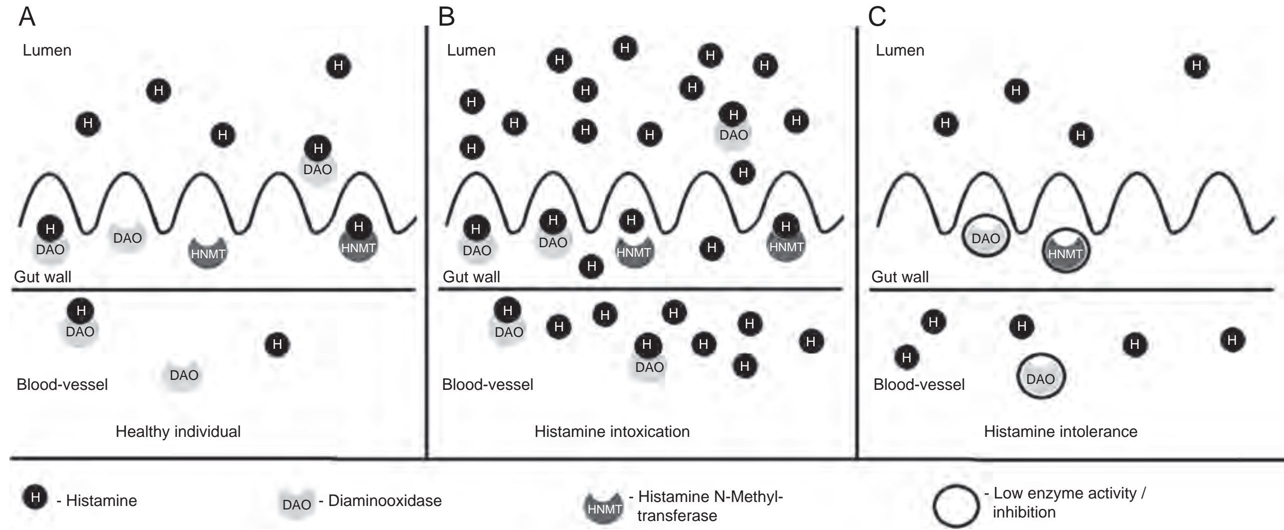

Figure 2 Degradation of histamine in gut. (A) Healthy individual. Normal concentration of histamine in food. Most histamine is inactivated by DAO and HNMT enzymes in gut, only a small amount of histamine passes to blood stream and does not cause histamine-mediated symptoms. (B) Histamine intoxication (scombroid poisoning). Ingestion of foods with high contents of histamine (more than 500 mg/kg) by healthy individual. Enzyme activity of DAO and HNMT is normal, but insufficient to inactivate excessive amount of histamine. Histamine passes to blood stream and evokes histamine-mediated symptoms. (C) Histamine intolerance. In individuals with histamine intolerance, enzyme activity of DAO and HNMT in gut is decreased or inhibited and insufficient to inactivate histamine from a food with normal concentration of histamine. Histamine passes to blood stream and evokes histamine-mediated symptoms.

lar histamine. On the other hand, HNMT is present in cytosol of cells and is able to degrade histamine only in intracellular space. In mammals, DAO expression is limited to specific tissues – the highest activity of DAO is present in small intestine and ascending colon, placenta and kidneys. Decrease of DAO activity can be a potential marker of damage of intestinal mucosa by inflammatory, malignant processes or by chemotherapy. HNMT is widely expressed in many human tissues – kidneys, liver, spleen, prostate, ovaries, in cells of spinal cord, in bronchi and the trachea.[8] Although both enzymes, DAO and HNMT, are present in intestinal epithelium, the main barrier of absorption of histamine into the blood stream is DAO. HNMT plays only a minor role in this process. Under normal circumstances, this enzymatic barrier sufficiently protects from resorption of histamine into blood stream (Fig. 2A).[15] Diamine oxidase is continuously secreted into the intestinal lumen. Therefore, in a healthy person histamine-rich food is largely eradicated of histamine in the intestine. The remaining quantity of histamine is degraded by DAO when it passes through the intestinal mucosa. DAO also protects the body from histamine formed physiologically by intestinal bacteria in the intestine.[16]

Histamine intoxication

Though histamine is quickly inactivated by diamine oxidase in healthy individuals, even in them severe symptoms resulting from increased concentration of histamine in blood can occur – histamine intoxication in case of ingestion of food with high contents of histamine, such as fish of fermented cheeses. Previous terms for histamine intoxication were scombroid fish poisoning, pseudoallergic fish poisoning, histamine overdose, or mahi-mahi flush. The term scombroid was used because the first fish species implicated in this poisoning were from the suborder *Scombridae*, which includes mackerel, tuna, marlin, swordfish, albacore, bonito, skipjack, and almost 100 other species (Scombridae is derived from the Greek word *scombros*, which means mackerel or tunny).[17] Not only scombroid fish, but also some non-scombroid fish (mahi mahi, bluefish, sardines, pilchards...) have high contents of histamine thanks to naturally occurring histidine, which is decarboxylated by the bacteria mentioned earlier in improper processing and storage of fish. Histamine is the only biogenic amine with regulatory limits set by legislation of the European Union, up to a maximum of 200 mg/kg in fresh fish and 400 mg/kg in fishery products treated by enzyme maturation in brine.[18] Pathogenesis of histamine intoxication cannot be explained by action of histamine alone. Scombrotoxic fish present with higher toxicity when compared to equivalent per oral dose of pure histamine. Other mechanisms are suggested, such as inhibition or potentiation of enzymes controlling histamine, presence of substances, which cause degranulation of mast cells, the presence of other histamine agonists and the existence of histamine intolerance, by which high interindividual variability in response to decomposed fish can be explained.[3] Toxicity of histamine can be potentiated by other substances produced in the process of decomposition of fish – cadaverin and putrescin, which inhibit intestinal inactivation enzymes mono- and diamine oxidases[19] or release histamine from bond to mucosal mucin and facilitate its absorption. Typical symptoms of histamine intoxication include rash, erythema, sweating, nausea, vomiting, diarrhoea, sensation of burning in mouth, swelling of tongue and face, headaches, respiratory distress, palpitations and hypotension. The symptoms of histamine poisoning can be present for few hours or a day, but in rare cases they may persist for several days. How-

ever, statistical data about its incidence are not available because the poisoning incidents are often underestimated due to mild or not recognised nature of illness and to inadequate systems to attribute food-borne diagnosis.[18] Diagnosis is confirmed by increased plasmatic concentration of histamine in the patient or in contaminated food.[20] In differential diagnosis from food allergy, the concentration of serum tryptase measured within 1–2 h after onset of symptoms can be helpful.[21] In food allergy, the activity of serum tryptase is increased and in histamine intoxication it is within physiological values. The course of histamine intoxication is usually mild, self-limited, lasts for short period of time and does not require therapy. In severe cases of histamine intoxication, therapeutic intervention is required – supportive volumotherapy and oxygenotherapy and oral antihistamines and bronchodilators are administered.

Histamine intolerance (HIT)

Histamine intolerance (abbreviated HIT) is a pathological process, in which due to the disproportion between intake of histamine and ability of the organism to eliminate it, excessive accumulation of histamine occurs and development of symptoms caused by its bond to corresponding receptors (H_1–H_4) (Fig. 2C). HIT has typically presented more often in people who are middle-aged and prevalence of it is estimated to be 1% of the population[22] although this diagnosis can be unrecognised and underestimated, because it manifests via the multi-faced clinical symptoms, which are often misinterpreted by the patient as well as by the physician. Sources of histamine and possible causes of its levels in organism are summarised in Table 1.

Under normal circumstances, there is an enzymatic barrier formed by DAO and HMNT in cells of intestinal epithelium in otherwise healthy individuals, which sufficiently protects from resorption of histamine from ingested food into the blood stream. Histamine intolerance, therefore increased histamine concentration in blood, can be caused when the amount of these protective enzymes is insufficient or these enzymes are inhibited.[15] In such cases, development of symptoms resulting from increased concentration of histamine occurs even in ingestion of small amount of histamine in food, which is usually well tolerated in healthy individuals.

Insufficient activity of DAO can occur based on genetic predisposition, in diseases of gastrointestinal tract, which decrease production of DAO by damaged enterocytes

Table 1 Sources of histamine and possible causes of increase of concentration in organism.

Naturally produced histamine, mostly in mast cells	
Naturally occurring histamine in larger quantities	Tomatoes, eggplant, spinach, fish, chicken and every stored meat All fermented food (cheeses, sausages, sauerkraut, wine, beer, champagne ...
Histamine liberators of natural origin	Pineapple, bananas, citrus fruits, strawberries, nuts, papaya, tomatoes, liquorice, spices, legumes, cocoa, alcohol; fish, seafood, pork, egg white
Histamine liberators of artificial origin	**Additives** (colourants, preservatives, stabilisers, taste enhancers, flavourings ...)
Bacteria and yeast contributing to histamine production	Foods with viable yeast – sourdough, fresh bread
Substances decreasing DAO activity in food	Alcohol
Substances decreasing DAO activity in medication	**Antiarrhythmics** (verapamil, propafenone), **antibiotics** (cefuroxime, cefotiame, acidum clavulanicum, doxycyclinum, isoniazid, framycetin), **painkillers** (metamizole), **antidepressants, psychiatric medication** (amitriptiline, diazepam, inhibitors MAO–1, haloperidol), **antiemetics** (metoclopramide), **antihistamines** (promethazine, cimetidine), **antihypertensive drugs** (dihydralazine), **antimalarials** (chloroquin), **bronchodilators** (aminophylline, theophylline), **diuretics** (furosemide), **mucolytics** (N-acetylcysteine, ambroxol), **muscle relaxant** (alcuronium, pancuronium, D-tubocurarin), **antiseptics** (acriflavinium chloride), chinidin
Histamine liberators in medication	**Painkillers** (morphine, pethidine, codeine, metamizole, **antiflogistics** (acetylsalicylic acid), **antibiotics** (D-cycloserine, chloroquin, pentamidine), **anti-hypotensives** (dobutamine), **antihypertensive drugs** (verapamil, alprenolol), **antitussives** (codeine), **cytostatics** (cyclophosphamide), **diuretics** (amilorid), **iodine-containing contrast medium**, **local anaesthetics** (mesocaine, procaine, marcaine, prilocaine), **muscle relaxant** (D-tubocurarin), **narcotics – anaesthetics** (barbiturates, thiopental)
Pyridoxine (vitamin B6) inactivating drugs	**Antihypertensive drugs** (hydralazine), **antibiotics** (D-cyklosporine, isoniazid), **hormonal contraception** (containing oestrogens)
Allergic reaction	IgE-mediated histamine release from mast cells
Substances potentiating allergic IgE-mediated histamine release	**Painkillers – antipyretics** (acetylsalicylic acid, diclofenac, flurbiprofen, indomethacin, ketoprofen, mefenamin, naproxen...)
Infection, trauma, shock	

Modified according to 29.

(inflammatory bowel diseases, infections, parasitic infestations, dysmicrobia, metabolic malabsorption),[23] or in inhibition of DAO by other biogenic amines, alcohol or medication. DAO gene polymorphisms significantly influence expression and activity of DAO, but they are not sufficient for the development of HIT on their own. Concurrence of environmental cofactors is of high importance, such as potential modifications of alternative histamine N-methyltransferase pathway, vesicular shift of both enzymes and amines or ability of enterocytes to reuptake histamine. Therefore, on the development of HIT there is a contribution of genetic, as well as environmental factors.[24]

Reduced DAO activity can be found in patients with chronic renal failure, viral hepatitis, advanced hepatic cirrhosis, and chronic urticaria – a typically histamine related illness with a reduced tolerance for endogenous histamine.[25]

Decreased degradation capacity of DAO can be caused by lack of its cofactors, vitamin B6, vitamin C, copper and zinc.[26] Some substances (histamine liberators) have the ability to release histamine from endogenous reserves in the organism.[10] Histamine can be synthesised from L-carnosine, which is released in the organism in physical activity and in stress in general. Dipeptide carnosine is present in tissues and is hydrolysed in stress, thus providing histamine. Studies realised in animal models suggest that in stress the concentration of carnosine decreases with a simultaneous increase of histamine concentration.[27]

Histamine concentration in the organism is also influenced by psychological stress. Hormones, which are released during stress reaction directly activate mast cells, which leads to the release of histamine and other inflammation factors.[28,63] Apart from that, stress has negative effects on epithelium of small intestine with proved influence on activity of membrane processes and increased permeability of this important barrier. This potentiates increase of input of histamine from intestine and its liberation from mast cells by CRH-dependent mechanisms.

Because the fact that histamine is an important mediator responsible for symptoms of classical allergy reaction type I – (IgE-mediated) hypersensitive reaction, it is difficult to differentiate this reaction from histamine intolerance, which has basically the same clinical manifestation. Unlike IgE-mediated food allergy, when even small amounts of ingested antigen lead to development of symptoms, in histamine intolerance the cumulative amount of ingested histamine plays the key role.[8]

Symptoms of HIT

Most symptoms of HIT develop primarily due to an increase in concentration of histamine in the organism. Secondary symptoms result from the fact that increased concentration of histamine stimulates synthesis and release of catecholamins, which can cause paradoxical increase of blood pressure (even though histamine itself causes its decrease), tachycardia, dysrhythmias, nervousness, sensation of inner tremor and sleep disturbances. Signs of HIT are summarised in Table 2.[29]

Symptoms of HIT – nervous system

Neurological symptoms of HIT include headaches. In patients diagnosed with migraine, increased plasmatic levels of histamine were reported not only during migraine attack, but also in asymptomatic stages of disease. In many such patients, HIT was confirmed based on decreased DAO activity. Foods rich in histamine (cheeses, wine) were triggers of headache. Limit of histamine intake in food led to disappearance of migraine symptoms.[30]

Nowadays we know that histamine may elicit, maintain, and aggravate headache, although the mechanisms for this are not completely understood. In some pathological processes (migraine, cluster headache, multiple sclerosis) an increased number of mast cells in brain was reported.[31] Histamine does not penetrate the blood–brain barrier (BBB); however, circulating histamine may influence hypothalamic activity via the circumventricular organs that lack BBB. The study of Levy et al.[32] corroborated that dural mast cell degranulation, which can be antagonised by capsaicin,[62]

Table 2 Symptoms of histamine intolerance.

Acute symptoms	
Skin	Itching, redness, hives, swelling
Gastrointestinal tract	Pain – cramps, bloating, diarrhoea, gastro-oesophageal reflux
Oral cavity, upper airways	Itching and swelling of the lips, tongue, Eustachian tube, glottis, sneezing, watery discharge, swelling of the lining of the nose, phlegm, cough
Lower airways	Cough, respiratory distress, asthmatic symptoms
Cardiovascular system	Changes in blood pressure (increased release of catecholamines results in large amounts of histamine), palpitations, heart rhythm disorders
Nervous system	Headache (dependent on movement – the position of the head, migraine) dizziness and loss of consciousness
Chronic symptoms	
Chronic inappropriate fatigue	
Dysmenorrhoea	
Nervousness, sleep disturbances (insomnia)	
Anxiety, panic disorder, depression	

Modified according to 29.

activates a pain pathway underlying migraine headache. Most antihistamines have been shown to be ineffective as acute medication for migraine. Two centrally acting potent H_1 receptor antagonists (cinnarizine and cyproheptadine) have been reported to be efficacious in preventing migraine.[33] H_1 receptor is vastly expressed on large intracranial arteries; it causes release of endothelial relaxation factor – NO.[34] However, efficacy of antihistamines has been ascribed other actions than antihistaminergic. In addition to H_1 receptors, other histamine receptor subtypes can be involved in pathophysiology of headaches. Activation of both the H_3 and the H_4 receptor promotes inhibitory actions on neurons. The H_3 receptor causes auto-inhibition of histaminergic neurons themselves, and centrally acting H_3 receptor agonist prodrugs have been shown to both inhibit neurogenic inflammation in dura, to induce sleep and to produce antinociception. Subcutaneous injections of N-alpha-methylhistamine, a catabolite of histamine with high affinity to the histamine H_3 receptor, probably have some migraine preventive effect.[35] A negative feedback on histamine release from mast cells in proximity to C-fibre endings has been a postulated mechanism. There are no registered ongoing studies on H_3 and H_4 receptor ligands in migraine.[33]

Brain histamine is synthesised by neurons that are restricted to the posterior basal hypothalamus, more specific to the tuberomamillary nucleus (TMN) which projects practically to the whole central nervous system. The posterior hypothalamus is the place in which several primary headaches originate. This area is initially involved in the prodromal phase of migraine attacks.[36]

The central histaminergic system plays an important role in the complex sleep–wake cycle, promoting cortical excitability during wakening and attention, and it consolidates the wake state. The period of the day, in the evenings and during the night, when there is reduced susceptibility for migraine attacks corresponds with less central histaminergic firing.[33]

Symptoms of HIT – gastrointestinal system

Apart from headache, other important symptoms of histamine intolerance are diffuse pain of stomach, colic, flatulence and diarrhoea. Increased concentration of histamine and decreased DAO activity were reported not only in histamine intolerance, but also in many inflammatory and cancer diseases of gastrointestinal tract (Crohn's disease, ulcerative colitis, allergic enteropathy, food allergy, colorectal cancer).[8] In intestinal mucosa of patients with food allergy, there is simultaneous decrease of HNMT. Enzymes DAO and HNMT cannot therefore compensate each other and total degradation capacity for histamine decreases.[37] DAO levels are decreased also in patients with anorexia nervosa, where malnutrition causes intestinal mucosal atrophy and damage.[38] On the other hand, histamine intolerance can mimic anorexia nervosa and due to similar symptoms (weight loss, diarrhoea, abdominal pain...) it can be misdiagnosed as anorexia nervosa. Accurate diagnosis and histamine-poor diet in such patients can lead to weight gain and improvement of all symptoms.[39]

Symptoms of HIT – respiratory tract

In patients with histamine intolerance, during or immediately after ingestion of foods with high content of histamine or alcohol symptoms such as rhinorrhoea, nasal obstruction and in extreme cases even attack of bronchial asthma, bronchoconstriction, coughing, wheezing with decrease in lung function can occur.[8,40] In patients with bronchial asthma the decreased activity of HNMT was reported.[41] HNMT is considered the key enzyme for histamine degradation in bronchial epithelium.[42]

Symptoms of HIT – skin

Decreased levels of DAO in serum, symptoms of HIT (chronic headaches, dysmenorrhoea, flush, gastrointestinal discomfort, intolerance of foods rich in histamine and alcohol) were observed in patients with atopic dermatitis significantly more often when compared with the control group. Diet with limited amounts of histamine led not only to suppression of symptoms of histamine intolerance, but mitigation of symptoms of atopic dermatitis as well in these patients.[26,43] Decreased activity of DAO was reported in patients with chronic urticaria as well, which is a disease mediated by histamine. Reduction of histamine in diet led to relief of urticaria symptoms.[44]

HIT and reproductive system

Women with histamine intolerance often suffer from dysmenorrhoea and headaches connected with menstrual cycle. These symptoms can be explained by mutual interaction between histamine and female sex hormones and its ability to support uterine contractions. Uterus contracts at the onset of menstruation. When the effect of histamine is at its highest, such cramps in the uterus may well be triggered by histamine. This hypothesis is confirmed by clinical observation that the administration of H_1 receptor blocker on the first day of menstruation may prevent pain.[45] Histamine by its bond to H_1 receptor dose-dependently stimulates synthesis of oestradiol and slightly influences synthesis of progesterone.[46] Oestradiol has the ability to stimulate, and progesterone to inhibit production of prostaglandin $PGF2\alpha$, which causes painful uterine contractions in primary dysmenorrhoea. The responsiveness of mast cells in relation to the menstrual cycle and their histamine release have also been investigated. Experiments in rats revealed high uterine histamine levels, mediated by oestradiol, and also greater uterine contractility, which might be a sign of the modulation of myometrial histamine receptors secondary to ovarian steroids.[45,47] Symptom intensity of histamine intolerance can vary based on phase of menstrual cycle, with mitigation during luteal phase, when the DAO level is the highest.[48]

Balance between histamine and DAO is necessary for uncomplicated course of pregnancy. Due to interaction with female sex hormones, vasoactive effects, and the ability to stimulate cell growth and proliferation, histamine plays an important role in interaction between embryo and uterus during pregnancy and substantially helps by process of placental development.[49] The placenta produces large quantities of DAO during pregnancy, which represents metabolic barrier preventing excessive amounts of biologically active histamine entering from the placenta into

maternal and foetal circulation. Concentration of DAO in pregnant women increases by 500 times in comparison with women who are not pregnant. Due to high placental DAO production in pregnant women with histamine intolerance the symptoms transitionally mitigate during pregnancy.[50] On the other hand, insufficient activity of placental DAO leads to many complications in pregnancy, such as diabetes, miscarriage and disturbances of trophoblast (mola hydatidosa, choriocarcinoma), premature rupture of foetal membranes and to premature births – these facts cause us to observe more pregnancy complications and more miscarriages in women with histamine intolerance.[51]

In addition, a role for histamine intolerance has been discussed in relation to sea sickness. In favour of an association is the similar risk profile (women, migraine patients), the predominantly histamine rich food at sea due to preserved foods and the therapeutic use of antihistamines.[25]

Diagnosis of HIT

Diagnosis of histamine intolerance is still quite difficult, because it manifests via the multi-faced histamine-mediated clinical symptoms (Table 3), which are often misinterpreted by the patient as well as physician and there is lack of a reliable biomarker for HIT diagnostics. These symptoms and their provocation by certain kinds of food, beverages and drugs are often attributed to different diseases, such as food allergy and other food intolerances, mastocytosis, psychosomatic diseases or adverse drug reactions. Potential food allergies should be excluded by skin prick test or by the determination of specific IgE for food allergens. Occult systemic mastocytosis should also be excluded, for example by measuring serum tryptase levels. Diagnosis of histamine intolerance requires the presentation of two or more typical symptoms of histamine intolerance.[52] The diagnostic algorithm of HIT should start with a diet accompanied by careful recording of symptoms which developed after ingestion of food, identification of the exact types of food which cause the symptoms to develop and determination of histamine content in those foods. Furthermore, it is necessary to rule out other potential sources of symptom development. Improvement following the introduction of a histamine-free diet can help aid in the diagnosis of a HIT. Since exposure to histamine exists beyond diet, total avoidance of histamines is not attainable.[53]

For determination of HIT diagnosis the use of double-blind placebo-controlled histamine food challenges with assessment of plasmatic histamine concentrations and objective assessment of symptoms developed by accumulated histamine have been proposed.[22] Besides its expense in daily practice, oral provocation with histamine is very difficult to standardise. It has been published that even in patients with overt histamine intolerance, oral provocation was positive in only 50% of those tested.[54] Komericki et al.[55] published a multicentre study on the non-reliability of blinded oral histamine provocation to confirm histamine intolerance.

Another diagnostic method is measurement of intestinal activity of DAO and HNMT and analysis of DAO and HNMT gene polymorphisms in order to determine possible genetic predisposition.[56] Determination of activity of histamine DAO and HNMT in peripheral blood is less credible, because plasmatic histamine concentration is very unstable and serum activity of DAO is decreased only in half of patients with HIT and in 17% of healthy control group.[24]

Kofler et al.[57] propose the use of so-called Histamine-50 skin-prick-test for diagnosis of HIT, which is histamine skin prick test with readings at 50 min. That study showed that patients with histamine intolerance and a control group do not remarkably differ in the size of their histamine wheals, but remarkably in their time course of the histamine wheals $\geq$3 mm read at different time points. This difference in skin prick test allows discriminating for histamine intolerance with sufficient sensitivity and specificity.

Table 3 Diagnosis of HIT.

Diagnosis of histamine intolerance
Diet diary – association of food consumption and symptoms
Identification of food causing symptoms
Determination of histamine content of symptom causing food
Exclusion of other causes (allergic, metabolic, toxic)
Double-blind, placebo-controlled oral histamine provocation in combination with determination of plasma histamine concentration and objective physical parameters (heart rate, blood pressure, erythema)
Determination of DAO and HMT content and activity in intestinal mucosa (not in peripheral blood plasma
Analysis of DAO and HMT genetic polymorphisms

Adapted from 56.

Therapy of HIT

The most effective therapy of histamine intolerance (Table 4) is limitation of foods rich in histamine. Selection of appropriate foods is often difficult, because manufacturers do not usually detail amounts of histamine in foods; it is therefore necessary to follow general suggestions. Because exposure to histamine exists beyond diet, total avoidance of histamines is not attainable.[22] Furthermore, it is important to limit intake of substances, which either directly cause or stimulate endogenous histamine release and inhibit activity of DAO and HNMT. Because intolerance to medications which interfere with histamine metabolism is common, these med-

Table 4 Therapy of histamine intolerance.

Therapy of histamine intolerance
Histamine receptor antagonists
Avoidance of histamine containing food (histamine content?)
Avoidance of substances inhibiting DAO and HMT
Avoidance of histamine releasing substances (endogenous histamine release)
DAO substitution (encapsulated pig kidney DAO)

Adapted from 29,56.

ications should be avoided if at all possible whenever they are suspected of playing a role in causing or maintaining histamine intolerance. Where their administration cannot be avoided, e.g. in studies using contrast media, or perioperatively, antihistamines and corticosteroids should be given prophylactically.[25]

Patients usually respond to a low-histamine diet in a few days, and the diet should be kept up for one month in responders (subjects no longer with symptoms); foods withdrawn are then gradually reintroduced one by one. Overall, fresh foods are advisable, whilst processed, preserved, and highly elaborated foods should be avoided. HIT is transient in many patients, who may go back to a normal diet. For more severe cases H_1 antihistamines such as dexchlorpheniramine and H_2 antihistamines such as ranitidine are recommended by some authors.[58] Nowadays, enzyme DAO can be per orally substituted by dietary supplements.[56] Supplementation of zinc, copper, vitamin C and vitamin B6, which act as cofactors for DAO, may also be administered to improve function. In the study by Hagel et al.,[59] an intravenous infusion of ascorbic acid decreased serum histamine concentrations in patients with pathologically increased histamine concentration.

In patients with most severe, practically every day manifestations of HIT, apart from diet and DAO supplementation the prevention of histamine-mediated reactions by H_1 antihistamines (such as dex-chlorpheniramine) and H_2 antihistamines (such as ranitidine) or cromolyn-derived medications which prevent mast cells degranulation are recommended by some authors.[58] In the case of HIT, these medications are effective only in higher dosage (twice as high as used in e.g. pollinosis) and they have to be taken continuously.[29]

Ethical disclosures

Patients' data protection. Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Protection of human subjects and animals in research. The authors declare that no experiments were performed on humans or animals for this investigation.

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POSTER PRESENTATION Open Access

Evaluation of the inhibitory effect of various drugs / active ingredients on the activity of human diamine oxidase *in vitro*

Roland Leitner[1], Eva Zoernpfenning[2], Albert Missbichler[3*]

From 6th Drug Hypersensitivity Meeting (DHM 6)
Bern, Switzerland. 9-12 April 2014

Background

In this study the influence of active ingredients of certain drugs on the activity of human diamine oxidase (DAO; EC 1.4.3.22) was quantified. DAO is the main enzyme in catabolism of biogenic amines in the intestine. Ingestion of food containing high amounts of biogenic amines in case of reduced activity of DAO leads to an accumulation of histamine which causes symptoms of histamine intolerance. Many drugs are suspected to inhibit DAO-activity, nevertheless, only few scientific data are available to support this thesis.

Method

Therefore, based on a selection of drugs / active ingredients by literature research, the interaction with purified human diamine oxidase is determined and quantified in vitro with an activity test. Various drugs at pharmacologic concentrations were incubated with human diamine oxidase. Inhibition of diamine oxidase activity was calculated as the percentage of inhibition versus control (no inhibitor). To exclude drug formulation specific influences active ingredients (AI) of drug products (D) in pure form were examined.

Results

Chloroquine and clavulanic acid showed greatest inhibition potential on diamine oxidase (> 90%). Cimetidine and verapamil showed inhibition of about 50%. Moderate influence on DAO was caused by isoniazid and metamizole, acetyl cysteine and amitriptyline (>20%). Diclofenac, metoclopramide, suxamethonium and thiamine have very low inhibition potential (<20%). Interestingly cyclophosphamide and ibuprofen displayed no effect on DAO.

Conclusion

Since even levels of about 30% inhibition may be critical, most of the observed substances, can be designated as DAO inhibitors. Other drug components than active ingredients did not affect DAO activity or its interaction with a specific drug.

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Histamine intolerance: a metabolic disease?
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Inflammation Research

SHORT COMMUNICATION

Histamine intolerance: a metabolic disease?

H. G. Schwelberger

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Abstract
Objective To evaluate the evidence regarding the disease concept of histamine intolerance as a state of inadequate histamine inactivation.
Methods Keyword-based systematic screening of the scientific literature and of public websites focusing on diagnostic and therapeutic procedures.
Results Histamine intolerance is commonly diagnosed based solely on subjective reporting of symptoms instead of following systematic diagnostic procedures based on objective laboratory and physical parameters. The only effective long-term therapy is avoidance of histamine-containing food.
Conclusions The concept of histamine intolerance as a metabolic disease is in need of more experimental and clinical evidence and affected patients will benefit from a clear, evidence-based diagnostic and therapeutic regime.

Keywords Histamine intolerance · Histamine metabolism · Diamine oxidase · Histamine N-methyltransferase · Diagnosis · Therapy

Introduction

Histamine intolerance describes a state where the catabolic capacity for endogenously released or exogenously administered histamine is insufficient leading to histamine mediated adverse reactions [1, 2]. Specifically, the terms histamine intolerance or enteral histaminosis are used to explain a variety of symptoms that appear to be caused by dietary histamine upon ingestion of food with a high histamine content, such as fish, cheese, meat products, and alcoholic beverages [3, 4]. The hype about this disease concept in the media and on numerous websites, especially in Central Europe, called for a critical evaluation of the current evidence regarding diagnostic procedures and therapeutic strategies.

Materials and methods

Scientific journal articles published on histamine intolerance were retrieved by a comprehensive medical subject heading and keyword search of the PubMed database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=PubMed) and evaluated for diagnostic and therapeutic approaches used. Additionally, the information presented on public websites retrieved by a Google search on "histamine intolerance" was screened for comprehensibility, accuracy, scientific foundation, and citation of sources.

Results and discussion

Histamine intolerance is thought to be caused mainly by ingestion of food containing high amounts of histamine by people with low intestinal histamine inactivation or inhibition of this activity by other food constituents or drugs, which leads to resorption of histamine in amounts sufficient for causing adverse reactions [1, 2]. Evaluation of more than 200 scientific journal articles and over 30 patient oriented websites dealing with this disease concept

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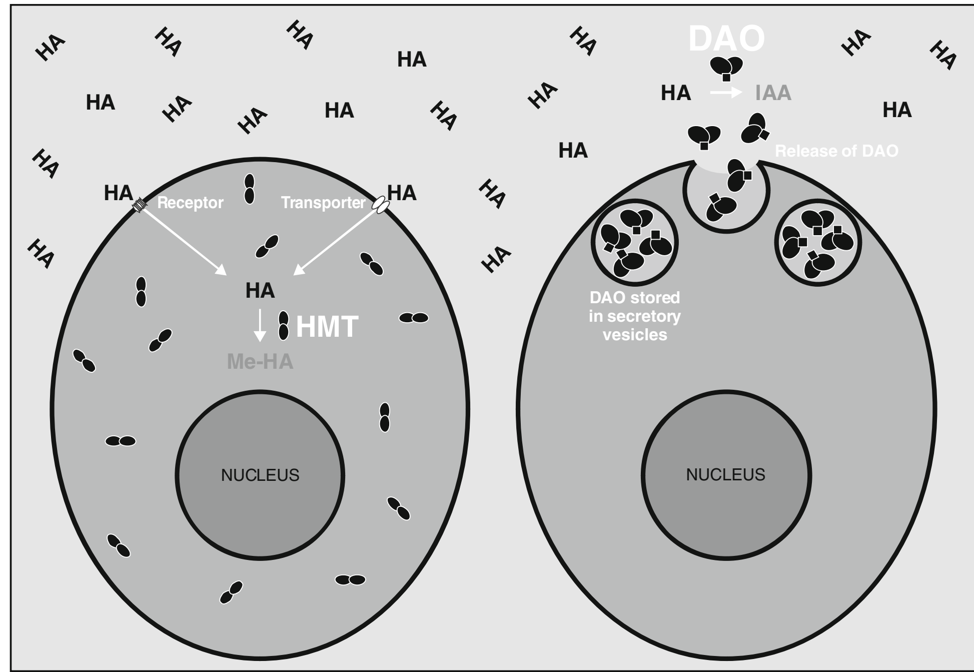

Fig. 1 Histamine (HA) is inactivated inside cells by histamine *N*-methyltransferase (HMT) yielding N^4-methylhistamine (Me-HA) and extracellularly by diamine oxidase (DAO) yielding imidazole acetaldehyde (IAA)

revealed that a lot more is being alleged and stated than is actually substantiated by scientific evidence.

Histamine present in food is usually associated with other pharmacologically active biogenic amines and is produced from the amino acid L-histidine by microorganisms possessing histidine decarboxylase activity in the course of food processing or spoilage [4]. For histamine to cause adverse reactions and symptoms it has to be resorbed in the intestine and transported via the bloodstream without being inactivated by the enzymes, diamine oxidase (DAO) and histamine N-methyltransferase (HMT), present in intestinal epithelial cells (Fig. 1). HMT is a cytosolic enzyme that inactivates histamine by methylation of the imidazole ring forming N^4-methylhistamine, which requires transport of histamine into the cell either by receptor mediated endocytosis or by specific transporters [5]. DAO is a secretory enzyme acting extracellularly that oxidatively deaminates the primary amino group of histamine yielding imidazole acetaldehyde and animal studies suggested that DAO forms the primary barrier for intestinal histamine resorption [6].

The major problem with the diagnosis of histamine intolerance is the variety of tests currently in use, ranging from the red wine provocation test to plasma DAO determination [1]. Adequate diagnosis of histamine intolerance (Fig. 2) should start with carefully recording symptoms

Fig. 2 Diagnosis and therapy of histamine intolerance

Diagnosis of histamine intolerance

- **Association of food consumption and symptoms (diet diary)**
- **Identification of food causing symptoms**
- **Determination of histamine content of symptom causing food**
- **Exclusion of other causes (allergic, metabolic, toxic)**
- **Double-blind, placebo-controlled oral histamine provocation in combination with determination of plasma histamine concentration and objective physical parameters (heart rate, blood pressure, erythema)**
- **Determination of DAO and HMT content and activity in intestinal mucosa (not in peripheral blood plasma)**
- **Analysis of DAO and HMT genetic polymorphisms**

Therapy of histamine intolerance

- **Histamine receptor antagonists**
- **Avoidance of histamine containing food (histamine content?)**
- **Avoidance of histamine releasing substances (endogenous histamine release)**
- **Avoidance of substances inhibiting DAO and HMT**
- **DAO substitution (encapsulated pig kidney DAO)**

after food consumption, identification of causative foodstuffs and determination of their histamine content. This will also be useful to either exclude or identify causes other than histamine. Definitive diagnosis necessitates double-blind, placebo-controlled oral histamine provocation with determination of plasma histamine concentrations and objective physical parameters. In proof of the concept, measurement of intestinal DAO and HMT activities is required and could be complemented by analysis of DAO and HMT gene polymorphisms to identify a possible genetic predisposition [7].

It is clear that therapy of histamine intolerance (Fig. 2) is useless without a firm diagnosis. Treatment with histamine H_1 and H_2 receptor antagonists is warranted only when uptake of high amounts of histamine occurs as in fish poisoning [8] but not for long-term therapy. The only effective therapy of confirmed histamine intolerance is avoidance of histamine containing food, which is difficult as the histamine content is usually not specified by producers and must be inferred from general recommendations [1]. Additionally, substances should be avoided that can either lead to endogenous histamine release or inhibit the activities of DAO and HMT. Controlled trials demonstrating the efficacy of DAO substitution with encapsulated pig kidney enzyme are still lacking. Even if the concept of histamine intolerance as a metabolic disease is in need of further experimental and clinical evidence, therapeutically any diet that improves the condition and does not lead to malnutrition will be beneficial for the patient.

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Histamine and histamine intolerance.

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Histamine and histamine intolerance[1–3]

Laura Maintz and Natalija Novak

ABSTRACT
Histamine intolerance results from a disequilibrium of accumulated histamine and the capacity for histamine degradation. Histamine is a biogenic amine that occurs to various degrees in many foods. In healthy persons, dietary histamine can be rapidly detoxified by amine oxidases, whereas persons with low amine oxidase activity are at risk of histamine toxicity. Diamine oxidase (DAO) is the main enzyme for the metabolism of ingested histamine. It has been proposed that DAO, when functioning as a secretory protein, may be responsible for scavenging extracellular histamine after mediator release. Conversely, histamine *N*-methyltransferase, the other important enzyme inactivating histamine, is a cytosolic protein that can convert histamine only in the intracellular space of cells. An impaired histamine degradation based on reduced DAO activity and the resulting histamine excess may cause numerous symptoms mimicking an allergic reaction. The ingestion of histamine-rich food or of alcohol or drugs that release histamine or block DAO may provoke diarrhea, headache, rhinoconjunctival symptoms, asthma, hypotension, arrhythmia, urticaria, pruritus, flushing, and other conditions in patients with histamine intolerance. Symptoms can be reduced by a histamine-free diet or be eliminated by antihistamines. However, because of the multifaceted nature of the symptoms, the existence of histamine intolerance has been underestimated, and further studies based on double-blind, placebo-controlled provocations are needed. In patients in whom the abovementioned symptoms are triggered by the corresponding substances and who have a negative diagnosis of allergy or internal disorders, histamine intolerance should be considered as an underlying pathomechanism. *Am J Clin Nutr* 2007;85:1185–96.

KEY WORDS Histamine intolerance, histamine, diamine oxidase, food intolerance, allergy

INTRODUCTION

Histamine intolerance results from a disequilibrium of accumulated histamine and the capacity for histamine degradation. The main enzyme for metabolism of ingested histamine is diamine oxidase (DAO) (1–5). An impaired histamine degradation based on a reduced DAO activity and the resulting excess of histamine may cause numerous symptoms mimicking an allergic reaction. Ingestion of histamine-rich food (6), alcohol (7–9), or drugs (10–13) that release histamine or block DAO may provoke diarrhea, headache (14), congestion of the nose, asthmatoid wheezing (6, 8, 15), hypotension, arrhythmia, urticaria (16, 17), pruritus, flushing, and other conditions in these patients. Approximately 1% of the population has histamine intolerance, and 80% of those patients are middle-aged (18). Because of the multifaceted symptoms, the existence of histamine intolerance is frequently underestimated, or its symptoms are misinterpreted. Clinical symptoms and their provocation by certain foods and beverages appear similar in different diseases, such as food allergy and intolerance of sulfites, histamine, or other biogenic amines (eg, tyramine). Therefore, the differentiation of the causal agent in adverse reactions to food, alcohol, and drugs is a difficult challenge. There is poor evidence of adverse reactions to these agents based on double-blind, placebo-controlled (DBPC) provocations (19). However, a better understanding of the pathophysiology, clinical picture, trigger factors, and diagnostic tools may help to clarify the confusing debate surrounding histamine intolerance.

HISTAMINE AND HISTAMINE METABOLISM

Histamine (2-[4-imidazolyl]ethylamine) was discovered in 1910 by Dale and Laidlaw (20), and it was identified as a mediator of anaphylactic reactions in 1932 (21). Histamine belongs to the biogenic amines and is synthesized by the pyridoxal phosphate (vitamin B-6)–containing L-histidine decarboxylase (HDC) from the amino acid histidine. It is synthesized by mast cells, basophils, platelets, histaminergic neurons, and enterochromaffine cells, where it is stored intracellularly in vesicles and released on stimulation. Histamine is a potent mediator of numerous biologic reactions. Besides the well-known triggering of degranulation of mast cells by crosslinking of the FcεRI receptor by specific allergens, several other nonimmunologic stimuli, such as neuropeptides, complement factors (ie, C3a and C5a), cytokines, hyperosmolarity, lipoproteins, adenosine, superoxidases (22), hypoxia, chemical and physical factors (eg, extreme temperatures, traumas) (23), or alcohol and certain food and drugs, may activate mast cells.

Histamine exerts its effects by binding to its 4 receptors [histamine 1 receptor (H1R), H2R, H3R, and and H4R] on target cells in various tissues (**Figure 1**, **Table 1**). It causes smooth

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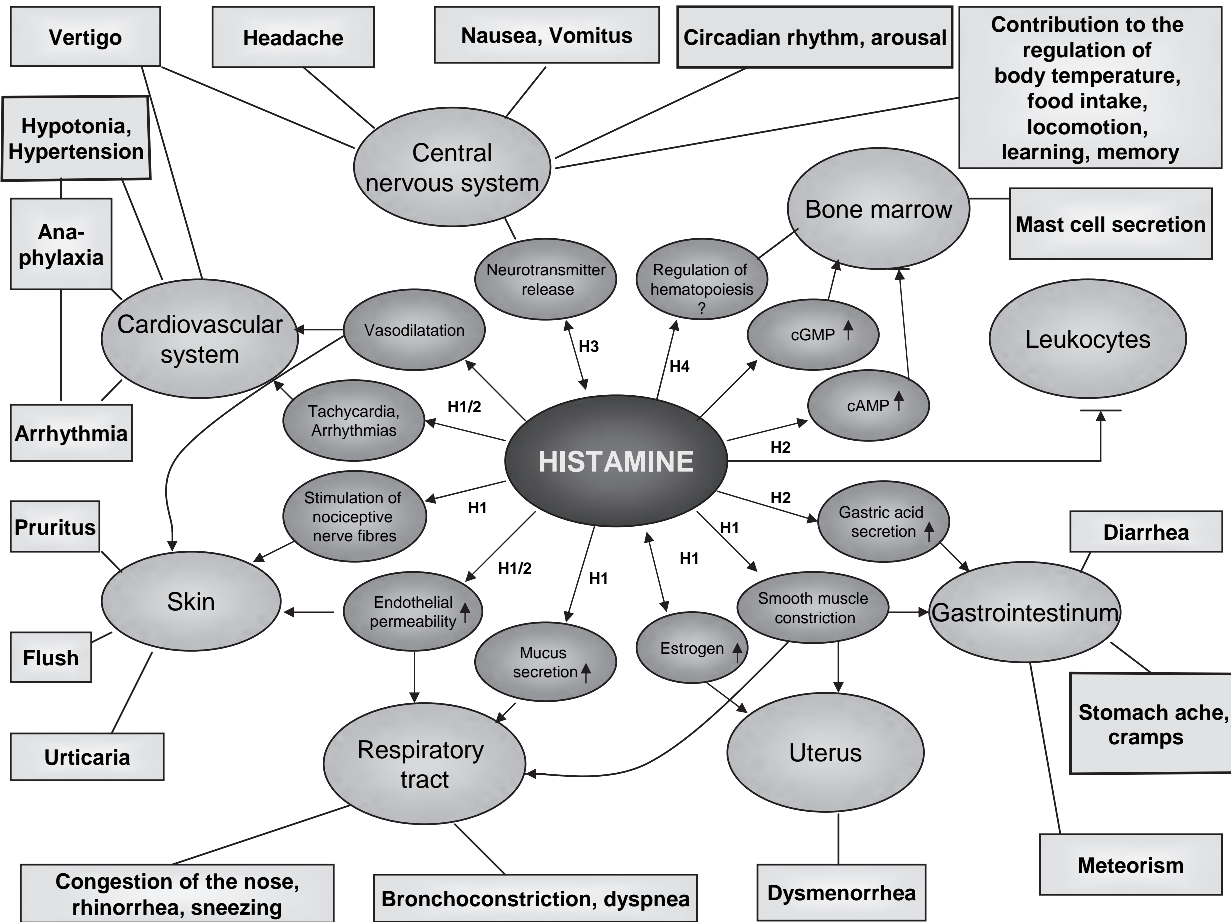

FIGURE 1. Summary of histamine-mediated symptoms. Adapted with permission from Maintz L et al. Dtsch Artzebl 2006;103:A3477-83.

muscle cell contraction, vasodilatation, increased vascular permeability and mucus secretion, tachycardia, alterations of blood pressure, and arrhythmias, and it stimulates gastric acid secretion and nociceptive nerve fibers. In addition, histamine has been known to play various roles in neurotransmission, immunomodulation, hematopoiesis, wound healing, day-night rhythm, and the regulation of histamine- and polyamine-induced cell proliferation and angiogenesis in tumor models (24, 25) and intestinal ischemia (26). Histamine can be metabolized in 2 ways: by oxidative deamination by DAO (former name: histaminase) or by ring methylation by histamine-*N*-methyltransferase (HNMT) (27) (**Figure 2**, **Table 2**). Whether histamine is catabolized by DAO or HNMT is supposed to depend on the localization of histamine. The DAO protein is stored in plasma membrane–associated vesicular structures in epithelial cells and is secreted into the circulation on stimulation (28, 29). Therefore, it has been proposed that DAO may be responsible for scavenging extracellular histamine (eg, after ingestion of histamine-rich food) after mediator release. Conversely, HNMT, the second most important enzyme inactivating histamine, is a cytosolic protein (30), which can convert histamine only in the intracellular space of cells (31, 32). Thus, the enzymes do not seem to compete for the substrate, although they have a similar affinity for histamine and they are expressed in some overlapping tissues. HNMT has a slightly higher affinity for histamine [Michaelis-Menten constant (k_M): 6–13 μmol/L] than does DAO (k_M: 20 μmol/L). In mammals, DAO expression is restricted to specific tissues; the highest activities are shown for small bowel and colon ascendens (4, 5, 33) and for placenta and kidney (28, 31). Lower DAO activity has been discussed as a potential indicator of intestinal mucosa damage in inflammatory and neoplastic diseases (17, 24, 34) and in persons undergoing chemotherapy (35). HNMT is widely expressed in human tissues; the greatest expression is in kidney and liver, followed by spleen, colon, prostate, ovary, spinal cord cells, bronchi, and trachea (36). HNMT is regarded as the key enzyme for histamine degradation in the bronchial epithelium (37).

TABLE 1
Histamine effects according to plasma histamine concentration (ng/mL)

Histamine	Clinical effect
0–1	Reference
1–2	↑ Gastric acid secretion ↑ Heart rate
3–5	Tachycardia, headache, flush, urticaria, pruritus
6–8	↓ Arterial pressure
7–12	Bronchospasm
≈100	Cardiac arrest

ETIOPATHOGENESIS OF HISTAMINE INTOLERANCE

Different mechanisms have been proposed as causing histamine intolerance (38). Histamine intolerance can develop

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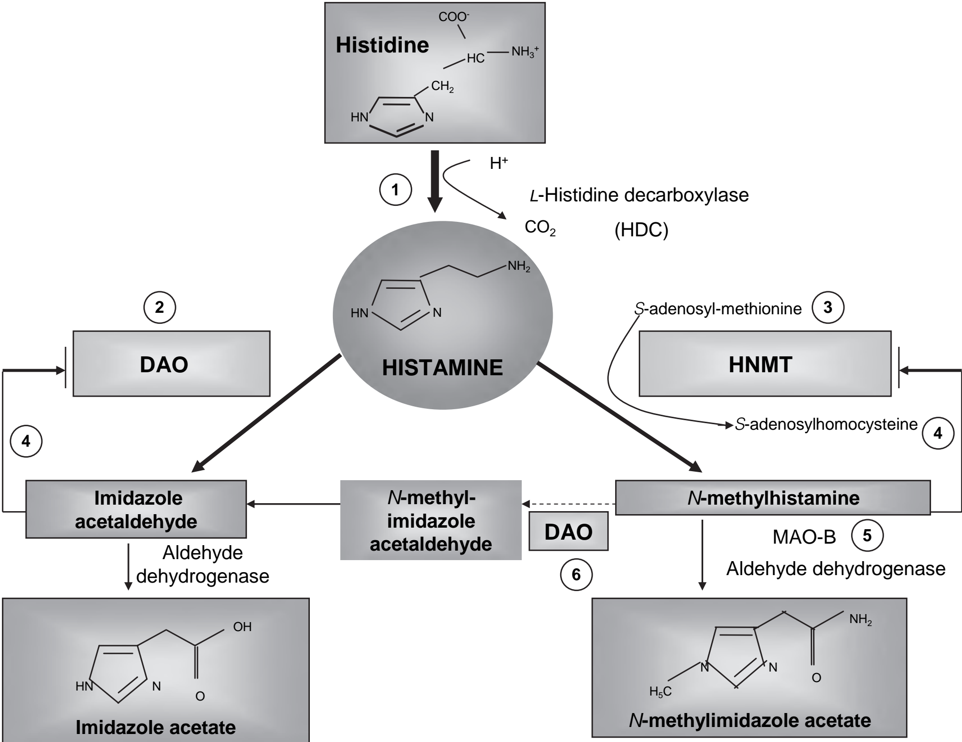

FIGURE 2. Summary of the histamine metabolism. The biogenic amine histamine is synthesized by decarboxylation of the amino acid histidine catalyzed by L-histidine decarboxylase (HDC) (1). Histamine can be metabolized by extracellular oxidative deamination of the primary amino group by diamine oxidase (DAO) (2) or intracellular methylation of the imidazole ring by histamine-*N*-methyltransferase (HNMT) (3). Therefore, insufficient enzyme activity caused by enzyme deficiency or inhibition may lead to accumulation of histamine. Both enzymes can be inhibited by their respective reaction products in a negative feedbackloop (4). *N*-Methylhistamine is oxidatively deaminated to *N*-methyl-imidazole acetaldehyde by monoamine oxidase B (MAO B) (5) or by DAO (6). Because the methylation pathway takes place in the cytosolic compartment of cells, MAO B (5) has been suggested to catalyze this reaction in vivo (35).

through both increased availability of histamine and impaired histamine degradation. Underlying conditions for increased availability may be an endogenous histamine overproduction caused by allergies, mastocytosis, bacterias, gastrointestinal bleeding, or increased exogenous ingestion of histidine or histamine by food or alcohol. Other biogenic amines, such as putrescine, may also be involved in displacing histamine from its mucosal mucine linkage, which results in an increase of free absorbable histamine in circulation. However, the main cause of histamine intolerance is an impaired enzymatic histamine degradation caused by genetic or acquired impairment of the enzymatic function of DAO or HNMT. Gastrointestinal diseases with altered enterocytes also may cause decreased production of DAO (17, 33, 39). Yet another cause can be competitive inhibition of histamine degradation of DAO by other biogenic amines, alcohol (7–9), or drugs (10, 12, 40). Acquired histamine intolerance may be transient and therefore reversible after the elimination of causes, such as by discontinuing DAO-blocking drugs. DAO inhibits the transepithelial permeation of exogenous histamine (41, 42), and impaired DAO activity results in increased enteral histamine uptake with consequent increased plasma histamine concentrations (10, 41) and corresponding symptoms. Increased amounts of histamine metabolites may also inhibit HNMT, the second enzyme metabolizing histamine (6, 43).

THE GENETIC BACKGROUND OF HISTAMINE INTOLERANCE

Recently, a potential genetic background of a reduced histamine metabolism has also been investigated. The human DAO gene spans ≈10 kbp and is located on chromosome 7q35 (27) Various single-nucleotide polymorphisms (SNPs) in the DAO gene have been shown to be associated with inflammatory and neoplastic gastrointestinal diseases, such as food allergy (44), gluten-sensitive enteropathy, Crohn disease, ulcerative colitis, and colon adenoma (45–47). No significant difference in the distribution of the investigated HNMT alleles could be shown between patients with gastrointestinal diseases and control subjects (45, 47), but a functional relevant polymorphism of the *HNMT* gene (chromosome 2q22) has been described for white asthma patients (48). Conversely, this association could not be observed in Japanese (49), German pediatric (50), and East Indian (51) populations. Thus, histamine intolerance seems to be acquired mostly through the impairment of DAO activity caused by gastrointestinal diseases or through the inhibition of DAO, but the high interindividual variations in the expression of DAO in the gut and the association of SNPs in the DAO gene with gastrointestinal diseases provide evidence for a genetic predisposition in a subgroup of patients with histamine intolerance (27).

CLINICAL PICTURE

Basal plasma histamine concentrations of 0.3 to 1.0 ng/mL are considered normal (52). Exceeding the individual histamine tolerance gives rise to concentration-dependent histamine-mediated symptoms (15, 53, 54) (Table 1). Even healthy persons may develop severe headache or flushing due to ingestion of massive amounts of histamine as is known from studies of scromboid poisoning (55). It has been shown that inhibition of

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TABLE 2
Characteristics of the histamine-degrading enzymes diamine oxidase (DAO) and histamine *N*-methyl-transferase (HNMT)[1]

	DAO	HNMT
Gene		
Gene map locus	Chromosome 7q35	Chromosome 2q22
Gene	10 kbp, 5 exons, 4 introns	35 kbp, 6 exons
Associated with SNPs	Inflammatory and neoplastic gastrointestinal diseases such as food allergy, gluten-sensitive enteropathy, Crohn disease, ulcerative colitis, and colon adenoma	Asthma
Protein	Soluble homodimeric glycoprotein of M_R 200 000 with subunits of 70–125 kDa; 750 amino acid residues	Soluble, cytosolic protein of M_R 33 000 with subunits of 29–34 kDa; 292 amino acid residues
Enzyme		
Group	Copper-containing amine oxidases	Methyltransferases
Active form	Homodimer with the active-site cofactor 2,4,5-trihydroxyphenylalanine quinone (Topa quinone)	Monomer with a 2-domain structure
Enzyme kinetics (k_m)	Histamine, 20 μmol/L Putrescine, 350 μmol/L Spermidine, 3 mmol/L	Histamine, 6–13 μmol/L *S*-adenosyl-L-methionine, 6–10 μmmol/L
Optimum pH	7.2	7.5–9.0
Inhibititors	Copper-chelating agents, eg cyanide Carbonylgroup reagents, eg, aminoguanidine, semibarbacide	Reaction products: *N*-methylhistamine, *S*-adenosyl-L-homocysteine Sulphydryl groups: p-chloromercuriobenzoate
Major expression	Intestine, kidney, placenta	Highest: kidney and liver; considerable: spleen, colon, prostate, ovary, spinal cord cells, trachea, and bronchi; to a smaller amount, nearly ubiquitous expression
Storage	Plasma membrane–associated vesicular structures in epithelial cells, secretion into the circulation upon stimulation	Cytosolic compartment of the cells
Function	Extracellular scavenger of histamine and other diamines by oxidative deamination of the primary amino group of histamine	Intracellular histamine inactivation by methylation of the imidazole ring

[1] SNPs, single-nucleotide polymorphisms; kbp, kilobase pair; M_R, molecular weight; kDa, kiloDalton; k_M, Michaelis-Menten constant.

DAO followed by oral histamine administration may induce severe and even life-threatening reactions, such as hypotension, bronchospasm, or shock (10, 43). Recurrent anaphylactic reactions have been reported in patients with hyperhistaminemia (56). In histamine-sensitive patients with reduced DAO activity, symptoms occur even after the ingestion of the small amounts of histamine that are well tolerated by healthy persons. Symptoms can be manifest via the abovementioned actions of histamine in multiple organs, such as the gastrointestinum, lung, skin, cardiovascular system, and brain, according to the expression of histamine receptors. Typical symptoms of histamine intolerance include gastrointestinal disorders, sneezing, rhinorrhea and congestion of the nose, headache (14, 57), dysmenorrhea, hypotonia, arrhythmias (58, 59), urticaria (16, 60), pruritus, flushing, and asthma (7, 8).

Histamine and headache

Headache can be induced dose-dependently by histamine in healthy persons as well as in patients with migraine (53, 61). Histamine-induced headache is a vascular headache caused mainly by nitrate monoxide (62). Histamine releases endothelial nitrate monoxide upon stimulation of H1R, which is also expressed in the large intracranial arteries (63). In migraine patients, plasma histamine concentrations have been shown to be elevated both during headache attacks and during symptom-free periods. An increase in the number of brain mast cells is associated with pathologic conditions such as migraine, cluster headache, and multiple sclerosis (64). Many migraine patients have histamine intolerance evidenced by reduced DAO activity, triggering of headache by food rich in histamine (eg, long-ripened cheese or wine), and the alleviation of headache (ie, disappearance of symptoms) under a histamine-free diet (57, 65) and therapy with antihistamines (66).

Histamine and gastrointestinum

Besides headache, gastrointestinal ailments including diffuse stomach ache, colic, flatulence, and diarrhea are leading symptoms of histamine intolerance. Elevated histamine concentrations and diminished DAO activities have been shown for various inflammatory and neoplastic diseases such as Crohn disease (17), ulcerative colitis (67), allergic enteropathy (39), food allergy (33, 68, 69), and colorectal neoplasmas (24). In the colonic mucosa of patients with food allergy, a concomitant reduced HNMT (70) and an impaired total histamine degradation capacity (THDC) (69) have been found (33), so that the enzymes cannot compensate each other. Therefore, an impaired histamine metabolism has been suggested to play a role in the pathogenesis of these diseases.

Histamine and airways

During or immediately after the ingestion of histamine-rich food or alcohol, rhinorrea or nasal obstruction may occur in patients with histamine intolerance; in extreme cases, asthma attacks also may occur. Reduced HNMT activity has been shown for patients with food allergy (70) and asthma bronchiale (71).

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TABLE 3
Foods rich in histamine[1]

Food categories	Histamine		Recommended upper limit for histamine		Tyramine	
	mg/kg	*mg/L*	*mg/kg*	*mg/L*	*mg/kg*	*mg/L*
Fish (frozen/smoked or salted/canned)			200		ND	
Mackerel	1–20/1–1788/ND–210					
Herring	1–4/5–121/1–479					
Sardine	ND/14–150/3–2000					
Tuna	ND/ND/1–402					
Cheese			No recommendation			
Gouda	10–900				10–900	
Camembert	0–1000				0–4000	
Cheddar	0–2100				0–1500	
Emmental	5–2500				0–700	
Swiss	4–2500				0–700	
Parmesan	10–581				0–840	
Meat			No recommendation			
Fermented sausage	ND–650				ND–1237	
Salami	1–654				-	
Fermented ham	38–271				123–618	
Vegetables						
Sauerkraut	0–229		10		2–951	
Spinach	30–60					
Eggplant	26					
Tomato ketchup	22					
Red wine vinegar	4					
Alcohol						
White wine		ND–10		2		1–8
Red wine		ND–30		2		ND–25
Top-fermented beer		ND–14				1.1–36.4
Bottom-fermented beer		ND–17				0.5–46.8
Champagne		670				

[1] ND, not detected. Data taken from references 13, 73, 75, 78, and 86.

Histamine and food

Histamine and other biogenic amines are present to various degrees in many foods, and their presence increases with maturation (1, 72). The formation of biogenic amines in food requires the availability of free amino acids, the presence of decarboxylase-positive microorganisms, and conditions allowing bacterial growth and decarboxylase activity. Free amino acids either occur as such in foods or may be liberated by proteolysis during processing or storage (73). Numerous bacterias and some yeast display high HDC activity and thus have the capacity to form histamine. Histidine is generated from autolytic or bacterial processes (74). Therefore, high concentrations of histamine are found mainly in products of microbial fermentation, such as aged cheese (75), sauerkraut, wine (76), and processed meat (77, 78) (**Table 3**) or in microbially spoiled food. Thus, histamine, tyramine, putrescine, and cadaverine serve as indicators of hygienic food quality (73). Tyramine and putrescine also may lead to intolerance reactions in combination with histamine. Possible explanations may be the inhibition of DAO by other amines (43) or the promotion of histamine liberation from the mucosa by putrescine (34).

Intolerance of tyramine that has vasoconstrictive properties that lead to hypertensive crisis and headache has been known mostly in patients taking monoamine oxidase (MAO)–inhibiting drugs. Orally administered tyramine in doses of 200 to 800 mg has been shown to increase systolic blood pressure by 30 mm Hg in otherwise unmedicated subjects. Conversely, in patients taking MAO-inhibiting drugs, the pressor sensitivity was 7- to 56-fold that in patients not taking MAO-inhibiting drugs (79). Eight DBPC studies have investigated the effect of tyramine on migraine. Two studies showed positive results in migraine patients who were sensitive to foods that are high in tyramine ($n = 45$) (19) or who had wine-provoked migraine ($n = 19$) (80); 6 studies showed negative results with 97 (81), 80 (82), 25 (83), and 65 (84) patients. The 2 positive studies and 2 of the negative studies were regarded as inconclusive (19) because of a lack of randomization (79), questionable blinding (80), or inappropriate selection of migraine patients without a history of suspected tyramine intolerance (81, 82). Conversely, in 2 conclusive studies of migraine patients with a positive or negative dietary history, 125 mg oral tyramine did not precipitate more headaches than did placebo.

In addition to histamine-rich food, many foods such as citrus foods are considered to have the capacity to release histamine directly from tissue mast cells, even if they themselves contain only small amounts of histamine (**Table 4**). In vitro studies of persons with a history of pseudoallergic reactions to food have shown a fragility of duodenal mast cells with massive degranulation in the presence of histamine-releasing substances that is significantly greater than that shown by control subjects (85). However, clinical studies using oral challenge tests to support the

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TABLE 4
Foods with suggested histamine-releasing capacities[1]

Plant-derived	Animal-derived	Other
Citrus fruit	Fish	Additives
Papaya	Crustaceans	Liquorice
Strawberries	Pork	Spices
Pineapple	Egg white	
Nuts		
Peanuts		
Tomatoes		
Spinach		
Chocolate		

[1] Data were taken from reference 21.

hypothesis for the histamine-releasing capacity of foods are required (22).

Alcohol, especially red wine, is rich in histamine and is a potent inhibitor of DAO (9, 86). The relation between the ingestion of wine, an increase in plasma histamine, and the occurrence of sneezing, flushing, headache, asthma attacks, and other anaphylactoid reactions and a reduction of symptoms by antihistamines has been shown in various studies (7, 8, 14, 65, 87, 88). However, among the multitude of substances contained in wine, other biogenic amines such as tyramine (80) and sulfites (89) have been supposed to contribute to symptoms summarized as "wine intolerance" or "red wine asthma" (19, 89, 90). In DBPC wine tests with healthy persons (91) and in patients with chronic urticaria and wine intolerance (92), the histamine content did not influence wine tolerance. In the latter group, an increase in plasma histamine could be shown, paradoxically, after ingestion of the histamine-poor wine. In these patients, the ethanol metabolite acetaldehyde has been discussed as a histamine-releasing substance (92). However, the high percentage of responses to the placebo (87%) could be responsible for the absence of an effect in this study (19). Another randomized DBPC oral wine challenge in patients with a history of red wine–provoked asthma ($n = 18$) found no relation between wine tolerance and the wine's content of histamine or other amines but did find a greater bronchoconstrictive response to wine with a high sulfite content (89). Sulfiting agents are widely used as antioxidants and preservatives in foods, beverages, and pharmaceuticals. Adverse reactions with a presumed relation to sulfites include anaphylactic shock, bronchospasm, urticaria, angioedema, nausea, abdominal pain, diarrhea, stroke, and death (93). Sulfite hypersensitivity has been reported mainly in patients with chronic asthma; the estimated prevalence is 5–10% in all patients (94). Asthmatic reactions have been attributed to reflex activation of the parasympathetic system by the irritating effect of sulfites, possibly enhanced by a deficiency of sulfite oxidase. Besides this pseudoallergic mechanism, in at least some cases of sulfite hypersensitity, an immunoglobulin E (IgE)–mediated immediate-type allergic reaction must be considered (95). Sulfites may be contained in wine, but they are also contained in foods that are poor in histamine, such as fruit juice, frozen vegetables, and lettuce. Thus, in patients reporting intolerance to wine, a careful history of reactions to other foods rich in histamine or sulfites should be taken. In patients who are suspected of having sulfite intolerance, skin testing and a DBPC challenge with capsules containing increasing doses of bisulfite or placebo should be performed.

In contrast to an IgE–mediated food allergy, in which the ingestion of even a small amount of the allergen elicits symptoms, in histamine intolerance, the cumulative amount of histamine is crucial. Besides variations in the amount of histamine in food according to storage and maturation, the quantity consumed, the presence of other biogenic amines, and the additional intake of alcohol or DAO-blocking drugs are pivotal factors in the tolerance of the ingested food. Generally, an upper limit of 100 mg histamine/kg in foods and of 2 mg histamine/L in alcoholic beverages has been suggested (96). This threshold may be too high, considering the occurrence of histamine-mediated symptoms after oral ingestion of 75 mg histamine in 5 of 10 females without a history of histamine intolerance (15).

However, most of the positive studies for intolerant reactions to sulfite, histamine, and other biogenic amines do not fulfill the current scientific criteria for providing substantiated evidence of the clinical effect of these foods. Nevertheless, patients who have a conclusive history of adverse reactions to food, alcohol, drugs containing histamine, other biogenic amines, and sulfite but without proof of IgE exist. In such patients, a DBPC provocation of the suspected causal agents under close supervision by experienced specialists should be performed after exclusion of other causal diseases and informed consent of the patients—if the provocation is not unreasonably hazardous, considering the grade of the anaphylactoid reaction. Because of the great effort, time, and costs or because of patients' fear of a repeated reaction, DBPC provocations often are not performed in clinical practice, even when they are indicated.

Histamine and drugs

The effect of drugs as specific DAO inhibitors and their capacity to induce histamine intolerance have been shown in various studies with human placental DAO and in animal experiments (10, 40, 97, 98). A clinically relevant activity via histamine release or inhibition of DAO has been observed for various drugs (10, 40, 97, 98) (**Table 5**). Therefore, the intake of drugs, especially long-term medication, should be considered in interpretation of histamine intolerance symptoms and DAO concentrations.

Other associated diseases

Reduced DAO activity—or, rather, reduced DAO release—after the application of heparin could be shown to be a marker of tissue damage in patients with chronic renal failure (99, 100), viral hepatitis (101), or gut failure and of endotoxemia in patients with liver cirrhosis (102). Reduced DAO activity has also been shown in patients with chronic urticaria as a typical histamine-mediated disease (60) combined with a reduced tolerance for infused histamine (16) and an improvement of urticaria by maintaining a histamine-free diet (103).

Histamine and atopic eczema

Higher basal plasma histamine concentrations (104, 105) and increased spontaneous histamine release toward different stimuli (106–108) and after food challenges (109) have been shown in patients with severe atopic eczema (AE) than in control subjects. In addition, reduced DAO activities have been shown in a subgroup of AE patients (104, 110, 111). Thus, these patients have a significantly greater occurrence of chronic headache, dysmenorrhea, flushing, gastrointestinal symptoms, and intolerance to

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TABLE 5
Drugs releasing histamine or inhibiting diamine oxidase

Substance class	Agent interfering with the histamine metabolism
Contrast media	
Muscle relaxants	Pancuronium, alcuronium, D-tubocurarine
Narcotics	Thiopental
Analgetics	Morphine, pethidine, nonsteroidal antiinflammatory drugs, acetylsalicylic acid, metamizole
Local anesthetics	Prilocaine
Antihypotonics	Dobutamine
Antihypertensive drugs	Verapamil, alprenolol, dihydralazine
Antiarrhythmics	Propafenone
Diuretics	Amiloride
Drugs influencing gut motility	Metoclopramide
Antibiotics	Cefuroxime, cefotiam, isoniazid, pentamidin, clavulanic acid, choroquine
Mucolytics	Acetylcysteine, ambroxol
Broncholytics	Aminophylline
H2-receptor antagonists	Cimetidine
Cytostatics	Cyclophosphamide
Antidepressants	Amitriptyline

alcohol and food than do control subjects. Reduction of both the symptoms of histamine intolerance and the severity score of atopic dermatitis (SCORAD) has been shown in a subgroup of patients with AE and low DAO serum activity who were following a histamine-free diet for 2 wk (111). Orally ingested histamine has been shown to aggravate eczema in AE patients in a DBPC provocation (112). A feedback inhibition of DAO through its degradation product imidazole acetic acid (113, 114) or substrate inhibition (115, 116) caused by the elevated histamine concentrations in AE may be a pathomechanism of a reduced histamine degradation capacity in a subgroup of patients with AE.

Histamine and sexual steroids

In the female genital tract, histamine is mainly produced by mast cells, endothelial cells, and epithelial cells in the uterus and ovaries. Histamine-intolerant women often suffer from headache that is dependent on their menstrual cycle and from dysmenorrhea. Besides the conctractile action of histamine, these symptoms may be explained by the interplay of histamine and hormones. Histamine has been shown to stimulate, in a dose-dependent manner, the synthesis of estradiol via H1R; meanwhile, only a moderate effect on progesterone synthesis was observed (117). The painful uterine contractions of primary dysmenorrhea are mainly caused by an increased mucosal production of prostaglandine F2α stimulated by estradiol and attenuated by progesterone. Thus, histamine may augment dysmenorrhea by increasing estrogen concentrations. And, in reverse, estrogen can influence histamine action. A significant increase in weal and flare size in response to histamine has been observed to correspond to ovulation and peak estrogen concentrations (118). In pregnancy, DAO is produced at very high concentrations by the placenta (119, 120), and its concentration may become 500 times that when the woman is not pregnant (120). This increased DAO production in pregnant women may be the reason why, in women with food intolerance, remissions frequently occur during pregnancy (14).

PRACTICAL CONSEQUENCES

Because of the multifaceted symptoms in multiple organs, a detailed history of the basal histamine-mediated symptoms, any triggering of symptoms after the intake of histamine-rich food or drugs interfering with the histamine metabolism, and concomitant gastrointestinal diseases or allergies is indispensable for diagnosis of histamine intolerance (**Figure 3**). Clinically, histamine-induced symptoms cannot always be assigned to the underlying pathomechanism. A massive intake of histamine from decomposed fish may result in the same symptoms as are seen in a person with an IgE-mediated fish allergy. Histamine actions may be possible causes of endogenous cell activation, increased exogenous uptake, decreased histamine degradation, or a combination of these mechanisms. An occult systemic mastocytosis should be excluded by measurement of the serum tryptase. Diagnosis of histamine intolerance is set by presentation of $\geq$2 typical symptoms of histamine intolerance (122) and improvement by histamine-free diet and antihistamines. The diagnosis of allergy using using the skin-prick test for food allergens or determination of specific IgE should be carried out to exclude food allergy. The diagnosis of allergy usually proves to be negative because histamine intolerance is a pseudoallergy. Keeping of a diet diary has proven useful in tracking significant improvement of symptoms with a histamine-free diet and relapses in histamine intolerance after dietary errors.

In a patient with clinical suspicion of histamine intolerance (ie, $\geq$2 typical symptoms), improvement of symptoms by histamine-free diet or antihistamines, DAO may be determined in serum (123) or tissue biopsy (32). Several radioextraction assays (REA) have been developed for the determination of the enzymatic activity of DAO by using [3H]- or [^{14}C]-labeled putrescinedihydrochloride as a substrate (124, 125). Determination of the HNMT activity is based on transmethylation of histamine by *S*-adenosyl-L [methyl-^{14}C] methionine (126). Furthermore, the total histamine degradation capacity can be measured (69). Plasma activity of DAO, which generally is relatively low, may be increased by the liberation of tissue-bound DAO through an injection of heparin (127–132), which was the main method used before the development of more sensitive assays. Serum DAO concentrations showed no significant daily variations and no significant sex differences (97). In patients with a DAO activity Histamine intolerance is presumably highly likely in patients with DAO activity $<$3 U/mL, likely (but less likely) in patients with DAO activity $<$10 U/mL, and improbable in patients with DAO activity $\geq$10 U/mL (18, 131).

Conversely, in some patients with a clear clinical picture of histamine intolerance, normal DAO activities have been observed, so that an additional determination of histamine concentrations and interpretation of laboratory data in view of the clinic seem advisable. Histamine can be measured in plasma or in urine, as can its degradation product *N*-methylhistamine (53, 132). Deficiency of the DAO cofactors vitamin B-6, copper, and vitamin C, which are thought to supplement histamine degradation (133), has been discussed as being controversial (14). Elevated histamine concentrations, reduced DAO activities, or both are classically found in histamine intolerance. A DBPC histamine provocation after a 4-wk histamine-free diet is considered the gold

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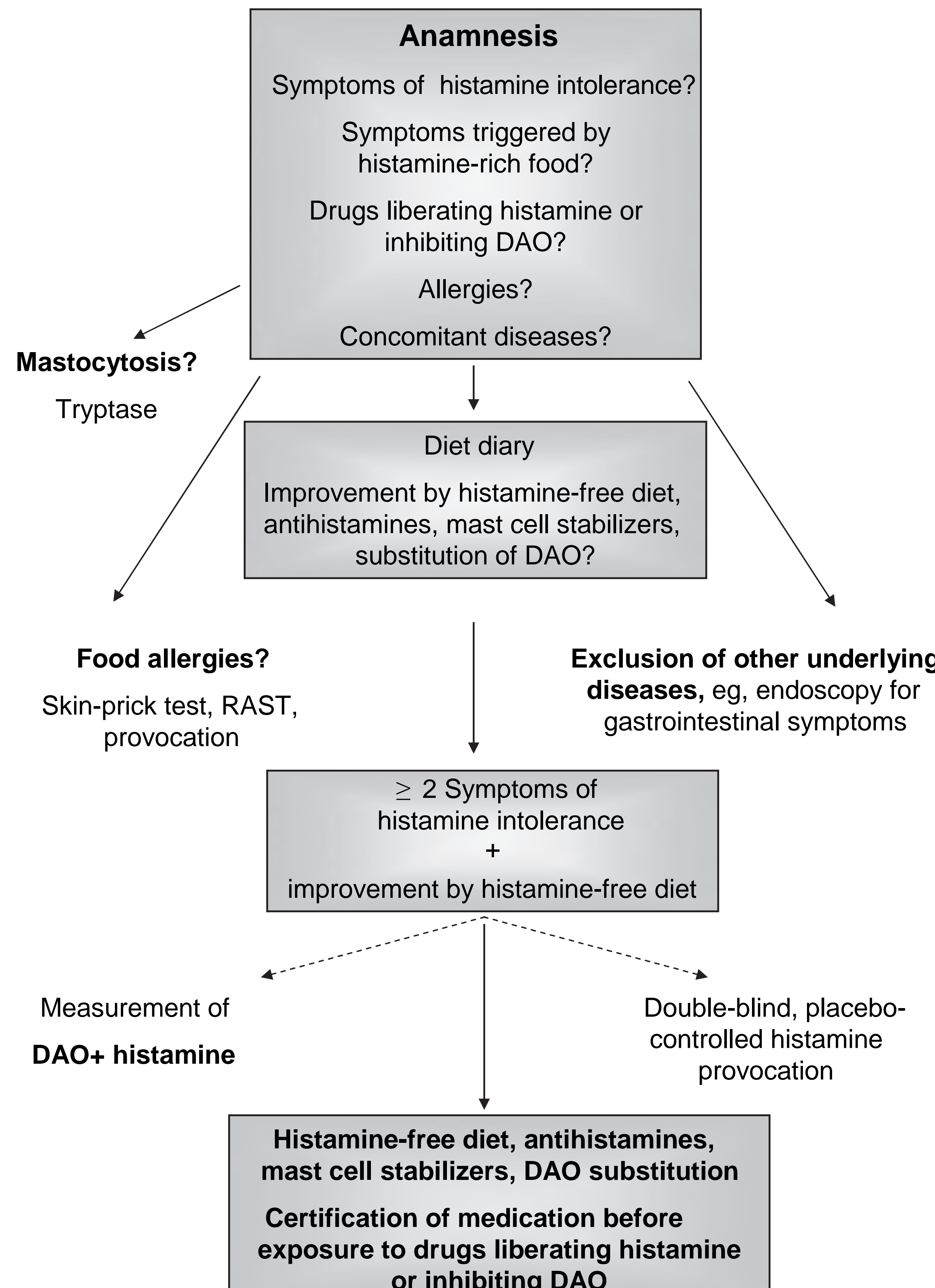

FIGURE 3. Diagnostic pathway for histamine intolerance. Adapted with permission from Maintz L et al. Dtsch Artzebl 2006;103:A3477-83.

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standard in diagnosis. Because the amount of histamine in natural food varies pronouncedly according to storage and maturation, the provocation can be performed with alternate administration of capsules containing increasing doses of histamine-dihydrochloride (0.75 and 1.5 mg/kg body wt, respectively) and placebo capsules (112). Blood pressure and heart rate should be continuously controlled, and positive reactions (eg, hypotonia, tachycardia, urticaria, or other symptoms of an anaphylactoid reaction) should be immediately treated by a physician. Afterward, symptoms should be evaluated by using a standardized symptom-scoring system.

Therapy is based on the consequent conduction of a histamine-free diet. Alcohol and long-ripened or fermented (and therefore histamine-rich) food, such as aged cheese, cured meat, and yeast products; histamine-rich food, such as spinach or tomatoes; or histamine liberators, such as citrus fruit, should be avoided (65, 134); the histamine-free diet can be complemented with adjuvant administration of H1 and H2 antagonists. Most antihistamines have no influence on DAO activity, although inhibition of DAO by cimetidine and dihydralazine and increased activity by diphenhydramine have been observed (97). In patients consuming a strictly histamine-free diet, no additional benefit due to an intake of antihistamines could be observed (57). An increase in DAO activity with the histamine-free diet was shown in migraine patients (57). In addition, histamine degradation can be supported by the administration of vitamin C (133) and vitamin B-6, which leads to an increase in DAO activity (14, 135). Positive effects have been reported for mast cell stabilizers and pancreatic enzymes (136), especially with respect to gastrointestinal symptoms. Because of the frequent intolerant reactions toward drugs that interfere with the histamine metabolism, their intake should be avoided. Recently, capsules containing DAO isolated from pig kidneys have been generated to supplement the lack of endogenous human DAO in patients with histamine intolerance. These capsules contain only stabilizers—ie, cellulose, sucrose, solanum tuberosum, polyacrylic acid, cellulose gum, triethyl citrate, and potato starch. Patients who are suspected of having histamine intolerance should be given a certificate noting that condition and stating that the administration of contrast and other drugs that release histamine should be avoided. If the administration of theses drugs is unavoidable (137), prior medication with antihistamines is recommended.

CONCLUSIONS

In patients with typical symptoms of histamine intolerance that are triggered by histamine-rich food and alcohol, with intolerance of drugs that liberate histamine or block DAO, and with a negative diagnosis of allergy or internal disorders, histamine intolerance should be considered. A histamine-free diet, if necessary, supported by antihistamines or the substitution of DAO, leads to an improvement of symptoms. However, further studies investigating histamine intolerance due to DBPC provocations are indispensable.

Both authors contributed equally in the literature review and the writing and editing of the article. Neither author had a personal or financial conflict of interest.

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ORIGINAL ARTICLE

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Human kidney diamine oxidase: heterologous expression, purification, and characterization

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Abstract Human kidney diamine oxidase has been overexpressed as a secreted enzyme under the control of a metallothionein promoter in *Drosophila* S2 cell culture. This represents the first heterologous overexpression and purification of a catalytically active, recombinant mammalian copper-containing amine oxidase. A rapid and highly efficient purification protocol using chromatography on heparin affinity, hydroxyapatite, and gel filtration media allows for the recovery of large quantities of the recombinant enzyme, which is judged to be greater than 98% homogenous by SDS/PAGE. The availability of large quantities of highly purified enzyme makes it now possible to investigate the spectroscopic, mechanistic, functional, and structural properties of this human enzyme at the molecular level. Visible absorption, circular dichroism, electron paramagnetic resonance, and resonance Raman spectroscopic results are presented. The recombinant enzyme contains the cofactors 2,4,5-trihydroxyphenylalaninequinone and copper at stoichiometries of up to 1.1 and 1.5 mol per mol homodimer, respectively. In addition, tightly bound and stoichiometric calcium ions were identified and proposed to occupy a second metal-binding site. The apparent molecular weight of the recombinant protein, determined by analytical ultracentrifugation, suggests 20–26% glycosylation by weight. Detailed kinetic studies indicate the preferred substrates (k_{cat}/K_M) of human diamine oxidase are, in order, histamine, 1-methylhistamine, and putrescine, with K_M values of 2.8, 3.4, and 20 μM, respectively. These results, demonstrating the substrate preference for histamine and 1-methylhistamine, were unanticipated given the available literature. The pH dependence of k_{cat} for putrescine oxidation gives two apparent pK_a values at 6.0 and 8.2. Tissue-specific expression of the human diamine oxidase gene was investigated using an mRNA array. The relevance of this work to earlier work and the suggested physiological roles of the human enzyme are discussed.

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Abbreviations *ABTS*: 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid · *CAO*: copper amine oxidase · *CHES*: 2-(*N*-cyclohexylamino)ethanesulfonic acid · *DAB*: *p*-dimethylaminomethylbenzylamine · *DAO*: diamine oxidase · *HEPES*: *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) · *hKDAO*: human kidney diamine oxidase · *MES*: 2-(*N*-morpholino) ethanesulfonic acid · *rhKDAO*: recombinant human kidney diamine oxidase · *TPQ*: 2,4,5-trihydroxyphenylalaninequinone

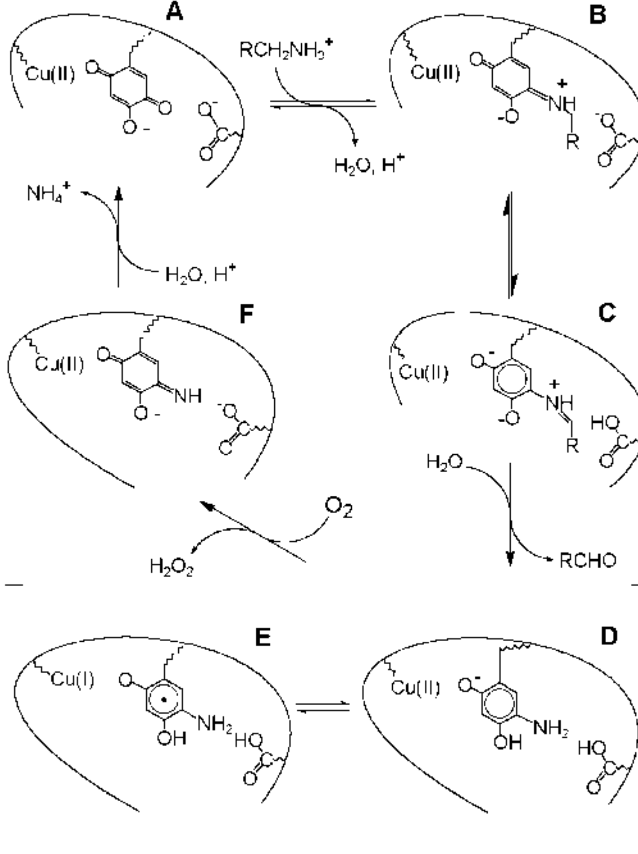
Introduction

Copper-containing amine oxidases (E.C.1.4.3.6) catalyze the two-electron oxidative deamination of primary amines to the corresponding aldehyde, using dioxygen as the oxidant, with the concomitant production of ammonia and hydrogen peroxide. Catalysis proceeds through a ping-pong mechanism divided into two half-reactions (Eqs. 1, 2, and Scheme 1):

$$E_{ox} + RCH_2NH_2 \rightleftarrows E - RCH_2NH_2 \rightarrow E_{red} + RCHO \quad (1)$$

$$E_{red} + O_2 \rightarrow E_{ox} + H_2O_2 + NH_3 \quad (2)$$

Copper-containing amine oxidases (CAOs) are widespread in Nature, having been found in bacteria, yeasts and fungi, plants and animals. These enzymes are homodimers, generally ranging in size from 140 to 200 kDa, with two active sites per dimer [1]. Each active



Scheme 1. Proposed reaction mechanism for the oxidation of amines by copper-containing amine oxidases. The oxidized, resting enzyme (**A**) combines with amine substrate to give a substrate Schiff base (**B**). Proton abstraction by the conserved active-site aspartate from the substrate's α-carbon results in a product Schiff base and reduced TPQ (**C**). Product aldehyde is release by hydrolysis, leaving the aminoquinol form of the enzyme (**D**). This Cu(II)-aminoquinol is in equilibrium with a Cu(I)-aminosemiquinone radical form (**E**). The enzyme is then oxidized, with dioxygen serving as the electron acceptor. Oxidation proceeds through a postulated aminoquinone intermediate (**F**), liberates ammonium and regenerates the resting enzyme (**A**)

site contains two cofactors: (1) a single type II copper ion, and (2) a quinone (2,4,5-trihydroxyphenylalanine-quinone, TPQ) derived from the post-translational modification of an invariant tyrosine residue [2]. TPQ has been shown to be produced in a novel, self-processing reaction requiring only copper and dioxygen [3, 4, 5, 6].

Three general classes of CAOs have been described from mammalian sources; unfortunately, the nomenclature in the literature is frequently confusing. One type of amine oxidase is found tightly associated with tissues, is active against monoamine substrates, and is commonly designated semicarbazide-sensitive amine oxidase (SSAO). It must be noted that all CAOs are inhibited by semicarbazide, so the designation of the tissue-associated amine oxidases as SSAOs is simply a convention. Sequence analysis has recently revealed these tissue-bound enzymes possess a single putative N-terminal transmembrane helix [7]. Another variety of CAO is soluble, found in blood plasma, and is active against a wide range of monoamines, diamines, and aromatic amines. These enzymes are generally termed plasma amine oxidases, serum amine oxidases, or benzylamine oxidases, and are likely synthesized in the liver [8]. The third type of mammalian CAO is also soluble, but displays distinct substrate specificity for diamines. This group is therefore termed diamine oxidases (DAOs). These are also distinct in sequence homology from the soluble plasma and the membrane-bound CAOs [9, 10, 11].

Barbry and co-workers [12] identified the first complete sequence for a mammalian copper-containing diamine oxidase, that of the human kidney (previously misidentified as a protein associated with the amiloride-sensitive Na^+ channel). The translated cDNA sequence encodes a 751 amino acid polypeptide with a predicted 19 amino acid signal sequence for the classical secretory pathway. N-terminal sequencing of human DAO purified from kidney and placenta demonstrated the mature protein lacked these residues, confirming the predicted signal cleavage site. The amino acid sequence contains the copper amine oxidase consensus sequence T/SXXNYD/EY/N (residues 457–463), in which the first tyrosine residue is modified to TPQ in the mature enzyme. A heparin-binding consensus sequence, RFKRRLPK, was also recognized (residues 569–576).

The structures of four copper amine oxidases have been solved by X-ray crystallography: two are bacterial (*Escherichia coli* and *Arthrobacter globiformis*), one is from the yeast *Hansenula polymorpha* (formally classified as *Pichia angusta*), and one is from pea seedling (*Pisum sativum*) [13, 14, 15, 16]. Collectively these four enzymes exhibit considerable structural homology, although primary sequence identity is less than 40% between any two. The enzymes are "mushroom" shaped with an extensive intersubunit contact, including a pair of "arms" extending from each subunit to embrace the other. Three or four domains are in each monomer, including an N-terminal domain that forms the mushroom "stalk" (not present in all CAOs) and a large, C-terminal β sandwich catalytic domain.

The crystallographically determined structures confirm the copper coordination environment as predicted from spectroscopy [17, 18, 19]. The active-site copper ion is coordinated in a distorted square pyramidal geometry by three conserved histidine residues and two water molecules, one axial and the other equatorial. Also, the quinone cofactor is in close proximity to the copper ion and is observed in different conformations, indicative of inherent side-chain flexibility.

One unanticipated finding in the crystal structures of the *E. coli* and pea seedling CAOs was the identification of an additional metal-binding site in each subunit, modeled as being occupied by a calcium and a manganese ion, respectively. Three aspartate carboxylates, two peptide carbonyl oxygens, and one water molecule coordinate this second metal ion. These aspartate ligands

are absolutely conserved in all 10 sequenced mammalian CAOs, as well as in 16 of 25 sequences representing bacteria, fungi, plants, and animals. Seven additional organisms retain two of the three aspartates. The second-metal site and the inferred location of the heparin binding sequence are both on the solvent-exposed upper surface of the "mushroom cap" [14].

To date, direct examination of the mammalian diamine oxidase has been very limited, especially for the human enzyme. We report herein the heterologous overexpression and purification of recombinant human kidney DAO, the first successful overexpression of any mammalian copper-containing amine oxidase. Furthermore, high expression levels and a purification protocol described herein provide rapid and efficient recovery of significant quantities of the highly purified enzyme, as required for detailed investigations of its molecular properties. Initial characterization of the recombinant enzyme includes molecular weight determination, cofactor quantification, and measurement of its visible absorption, circular dichroism, electron paramagnetic resonance, and resonance Raman spectra. Steady-state kinetic parameters, pH dependence, and substrate specificity for the recombinant enzyme are reported. A tissue-specific expression profile for the human enzyme is presented, as is an analysis of the biological relevance of the enzyme.

Materials and methods

Expression cell line

The coding sequence for mature human kidney diamine oxidase was amplified by PCR from a cDNA clone (kindly provided by Dr. Barbry, Institut de Pharmacologie Moléculaire et Cellulaire, France) using Vent DNA Polymerase and appropriate primer adapters. The forward primer (5′-GTGAGATCTCCGGG-GACTCTGCCC) replaced the N-terminal codons for glutamic acid and proline of mature kidney DAO with the codon for arginine and introduced a *Bgl*II site. The reverse primer (5′-CCGGAATTCACGATGCCGGCCCTGGGCTGGGCC) introduced an *Eco*RI site just downstream from the native stop codon. The PCR product and the expression vector pMT/BiP/V5-His A (Invitrogen) were digested with *Bgl*II and *Eco*RI, agarose gel purified, recovered using Prep-A-Gene (Bio-Rad), and ligated with T4 DNA ligase. The 5′ end of the resulting construct was confirmed by DNA sequencing (Silver Sequence, Promega) through the fusion site to an internal *Bst*II site, 350 base pairs into the coding sequence. The remainder of the coding sequence was swapped with a *Bst*II and *Eco*RI fragment from the cDNA clone to generate the expression vector pMTDAO. All DNA manipulations used enzymes from New England Biolabs. Constructs were maintained in *E. coli* strain TOP10 and purified using either Perfect Prep (5Prime3Prime) or Quantum Prep (Bio-Rad).

Transfection and cell culturing procedures were those outlined in the *Drosophila* Expression System Manual (Invitrogen), except as noted. Plasmids pMTDAO and the selection vector pCoHY-GRO were cotransfected into *Drosophila* Schneider 2 (S2) cells at a ratio of 19:1 (μg) using the Calcium Phosphate Transfection Kit (Invitrogen). Selection for the stably transfected subpopulation used 500 μg mL^{-1} hygromycin B (Roche Molecular Biochemicals). The resulting polyclonal cell line was adapted to and maintained in a serum-free medium (Ex-Cell 400, JRH Biosciences) supplemented with 300 μg mL^{-1} hygromycin B at 27 °C.

Expression and purification

The transfected cell line was expanded from a 5 mL culture in a 25 cm^2 tissue culture flask to a single 130 mL culture in a 250 mL spinner flask. When the spinner culture reached a density of 1×10^7 cells mL^{-1}, 25 mL of the culture was added to each of four 500 mL baffled shake flasks (Bellco Glass) containing 150 mL serum-free medium. The flasks were incubated in a gyrotary water bath at 110 rpm and 27 °C for about 24 h. At a density of 5×10^6 cells mL^{-1}, expression was induced by addition of copper sulfate to a final concentration of 500 μM, and incubation was then continued for 48 h. Nontransfected *Drosophila* S2 cells and the uninduced expression cell line were used as negative expression controls.

Cultures were harvested and cells spun out by centrifugation for 2 min at 1000×*g*. The supernatant was spun for 10 min at 10,000×*g* to remove particulates and then loaded on a 5 mL HiTrap Heparin HP column (Amersham Pharmacia Biotech) using a peristaltic pump. The column was then washed with 100 mM potassium phosphate buffer, pH 7.2, until A_{280} of the flow through reached zero, and bound protein was eluted with 100 mM potassium phosphate with 1 M sodium chloride, pH 7.2. The eluant was extensively dialyzed against 100 mM potassium phosphate, pH 7.2, and then loaded on Macro-Prep Ceramic Hydroxyapatite (type I, 40 μm particle size, Bio-Rad) in a HR 10/10 column using a FPLC system (Amersham Pharmacia Biotech). Buffers for the ceramic hydroxyapatite column were 100 mM potassium phosphate, pH 7.2 (buffer A) and 400 mM potassium phosphate, pH 7.2 (buffer B). Protein fractions were eluted with a two-column volume wash at 25% buffer B, a single column volume wash at 35% buffer B, and a two-column volume linear gradient from 35% to 100% buffer B. The most active fractions were pooled and concentrated in a 50 mL centrifugal concentrator (Millipore) before being run over a 1.6×100 cm Ultrogel AcA 34 (BioSepra) gel filtration column equilibrated in 100 mM potassium phosphate, pH 7.2. SDS/PAGE and IEF gels were by a PhastSystem (Pharmacia). Substantial absorbance at 280 nm in the culture media necessitated that initial protein concentration be determination by the Bradford protein assay (Bio-Rad) with bovine serum albumin standards. Subsequent protein concentrations were determined spectrophotometrically by absorbance at 280 nm using the predicted extinction coefficient for the mature, recombinant enzyme of 280.5 mM^{-1} cm^{-1} [20]. The extinction coefficient at 280 nm was later determined by magnetic circular dichroism and calculated as 297.6 mM^{-1} cm^{-1} (data not shown) [21].

Amine oxidase activity was measured at 37 °C in a stirred, thermostatted cuvette using a coupled assay with putrescine (dihydrochloride, Sigma) as the substrate. Assays used 30 U horseradish peroxidase (Sigma), 10 mM putrescine, and 2 mM ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] in 100 mM potassium phosphate, pH 7.2, in a final volume of 2 mL. Reaction progress was followed spectrophotometrically by monitoring the change in absorbance at 414 nm ($\epsilon = 24.6$ mM^{-1} cm^{-1}) [22]. Activities measured by dioxygen depletion using an Instech oxygen electrode and chamber were in close agreement to those measured by the coupled assay (data not shown).

General characterization

UV and visible absorption data were acquired with either a Hewlett-Packard 8452A or 8453 diode-array spectrophotometer. CD spectra were obtained with a Jasco J-710 spectropolarimeter. Titrations with phenylhydrazine (HCl, Sigma) were used to quantify TPQ in the purified recombinant enzyme [23]. One mL of 10–20 μM protein in 100 mM potassium phosphate buffer was titrated with 2 μL aliquots of fresh, anaerobically prepared phenylhdrazine (~775 μM) at room temperature. Spectral changes were monitored and recorded after the absorbance at 445 nm reached a constant value following each addition (10–45 min). The derivatized enzyme was subsequently concentrated in a Microcon 30 (Millipore) with buffer exchange to remove unreacted phenylhydrazine. The concentrated phenylhydrazine-derivatized enzyme was then used for

resonance Raman spectroscopy on a Spex Triplemate spectrometer with a CCD detector, with excitation by a Coherent argon-ion laser. EPR spectra were recorded on a Bruker 220D SRC interfaced with a personal computer. Simulation of EPR spectra used the DOS EPR spectra manipulation program EPPER from Professor John Lipscomb (University of Minnesota), which incorporates the simulation program EPRGHA [24]. Copper, zinc, calcium, and magnesium analyses were performed by ICP emission spectroscopy (Little Bear Laboratories, Golden, Co.), or copper analysis by flame atomic absorption spectroscopy using a Buck Scientific model 210 VGP. Metal-free buffer was prepared by passage over Chelex-100 (Bio-Rad) and by using plasticware treated with 0.1 M EDTA solution. Analytical ultracentrifugation and ES/MS services were kindly provided by Andy Baron and Alison Ashcroft, respectively, at the University of Leeds, Leeds, UK.

Steady-state kinetics

K_M and k_{cat} were determined spectrophotometrically in a 37 °C thermostatted cell holder with magnetic stirring. Ionic strength of the assay buffer, 50 mM HEPES, pH 7.2, was maintained at 150 mM by addition of potassium chloride. Oxidation of *p*-dimethylaminomethylbenzylamine (DAB) and benzylamine were followed by the change in absorbance at 250 nm, using an extinction coefficient of 11 $mM^{-1}\,cm^{-1}$ for *p*-dimethylaminomethylbenzaldehyde [25]. Assays with DAB or benzylamine used 890 µL of assay buffer and 10 µL enzyme in a 1 cm pathlength cuvette. This mixture was allowed to equilibrate for 2–3 min in the thermostatted cell holder before rapid addition of 100 µL substrate stock solution. Data acquisition was initiated immediately. All other assays used the coupled assay of Holt et al. [26] and an extinction coefficient for the quinoneimine dye of 6.00 $mM^{-1}\,cm^{-1}$ at 498 nm [27]. Chromogen stock solution was prepared to give final concentrations of 1 mM 4-aminoantipyrine and 2 mM vanillic acid. Coupled assays used 880 µL chromogen stock solution, 10 µL (14.5 U) horseradish peroxidase, and 10 µL enzyme. Substrate, horseradish peroxidase, and chromogen stock solutions were freshly prepared in the assay buffer, and kept in a 37 °C water bath until use. Recombinant DAO was kept on ice. Thermal equilibration, substrate addition, and data acquisition were as described above. Assays used 10 µL of 4.37 µM recombinant human kidney DAO, or 4.09 µM enzyme in the case of 1-methylhistamine. Water bath and thermostatted cell holder temperatures were monitored with an electronic thermocouple. Initial rates were determined by at least duplicate experiments (most often triplicate) at six or more substrate concentrations and fitting to the Michaelis-Menten equation using Origin software (OriginLab). Those substrates demonstrating substrate inhibition were also fit to Eq. 3 [28]:

$$v = V_{max}[S] / \left(K_M + [S] + [S]^2/K_i\right) \quad (3)$$

Substrates that oxidized either extremely slowly or at rates undetectable under these conditions were assayed up to millimolar substrate concentrations. Potential substrates were purchased commercially (highest grade available) and used without further purification, except for DAB, which was synthesized by the method of Bardsley et al. [25].

The pH dependence of Michaelis-Menten parameters for the oxidation of putrescine was determined using the coupled 4-aminoantipyrine/vanillic acid assay as described above. Buffers used were 50 mM MES (pH 5.69–6.33), 50 mM HEPES (pH 6.33–7.78), and 50 mM CHES (pH 8.12 and 8.85). Ionic strength was adjusted to a final concentration of 150 mM with potassium chloride. After equilibration at 37 °C, the pH was measured with an Orion perpHect LogR meter, model 310, equipped with an Automatic Temperature Compensation Probe. At least two (usually three or four) initial rates were determined at six or more substrate concentrations for each pH value. k_{cat} values were plotted against proton concentration and fit to the following equation [29]:

$$k_{cat} = (k_{cat})_{max}[H^+]pK_{a1} / \left(pK_{a1}pK_{a2} + pK_{a1}[H^+] + [H^+]^2\right) \quad (4)$$

Enzyme stability in the pH range used was tested by incubation of the enzyme in the appropriate buffers for 15 min at 37 °C and then measuring the activity. Possible effects of pH on the quinoleimine dye generated during the assay were investigated by titrating hydrogen peroxide into the chromogenic solution plus horseradish peroxidase at appropriate pH values. Neither loss of enzyme activity nor change in quinoleimine dye absorption features was detected in the buffers and pH range investigated.

Heparin effects on enzyme activity were investigated by incubating recombinant kidney DAO with an approximately four-fold excess of heparin (from porcine intestinal mucosa, average molecular weight of 3000 Da, Sigma) over dimeric protein for 1 h on ice. Initial rate determinations for putrescine oxidation were as described above. The enzyme-heparin mix was stored at 4 °C and assayed for activity with 250 µM putrescine after 24, 48, and 72 h. All assays used air-saturated solutions and dissolved oxygen levels were not varied in these experiments (about 233 µM).

Tissue-specific gene expression

A Multiple Tissue Expression Array (Clontech) was used to determine the human, tissue-specific expression profile of human DAO. The manufacturer's instructions were followed for generating ^{35}P-labeled cDNA probes by random primer labeling, hybridization, and autoradiography.

Results

Expression and purification

The expression vector pMTDAO contains the coding sequence for mature human kidney diamine oxidase (hKDAO) fused to the *Drosophila* BiP signal sequence for secretion from *Drosophila* S2 cell culture. Expression is under the control of the *Drosophila* metallothionein promoter and induced by addition of copper sulfate to 500 µM. Mature recombinant human kidney diamine oxidase (rhKDAO) primary sequence, as deduced from the DNA sequence, differs from that of the natural protein in only the replacement of the N-terminal glutamic acid and proline with an arginine residue.

Culture media was harvested 48 h post-induction at a cell density of 1.45×10^7 cells mL^{-1}. Cells were greater than 99% viable, as determined by trypan blue staining, and exhibited normal morphology. No amine oxidase activity was detected in either the uninduced expression cell line or in the parental S2 cell line.

The recombinant enzyme is readily purified by heparin affinity chromatography, ceramic hydroxyapatite chromatography, and gel filtration; the summary of a typical purification is given in Table 1. Total enzymatic activity increases during the protocol and likely reflects the loss of an inhibiting substance (of either the amine oxidase or the coupled assay) that is present in the growth medium. The purified protein is estimated at greater than 98% homogeneous by SDS/PAGE, and the highest specific activity obtained from any purification was 1.25 IU mg^{-1}.

Table 1. Purification of recombinant human kidney diamine oxidase

Step	Total volume (mL)	Total protein (mg)	Total activity (IU)	Specific activity (IU mg^{-1})	Purification factor
Culture medium	630	117	7.3	0.063	1
Hi-Trap Heparin	13.5	41	12.5	0.30	4.8
Ceramic hydroxyapatite	44	14	13.0	0.93	14.8
Ultrogel AcA34	0.74	12	12.7	1.1	16.7

Fig. 1. SDS/PAGE showing purification of recombinant human kidney diamine oxidase. *Lane 1*: harvested *Drosophila* S2 media diluted 1:1 with gel loading buffer. *Lane 2*: molecular weight standards: 94, 67, 43, 30, 20.1, and 14.4 kDa. *Lane 3*: protein after Hi-Trap Heparin affinity column, 3.6 µg. *Lane 4*: following ceramic hydroxyapatite chromatography. *Lane 5*: purified protein after gel filtration chromatography, 2.4 µg. *Lane 6*: 9.6 µg of the rhKDAO. The distorted band is a consequence of the severe overloading of the gel; however, this lane demonstrates the high degree of purity for the recombinant enzyme

Purified rhKDAO is stable for several months when stored on ice. However, freezing the enzyme at –20 °C gives a significant drop in specific activity; thawed enzyme has about 40% the specific activity of that before freezing. Thawed protein can recover 85% of the original specific activity after storing on ice for 10 days. Bieganski and co-workers [30] have previously noted a 90% loss in activity with natural human DAO when stored at –20 °C.

Electrophoresis and analytical ultracentrifugation

Isoelectric focusing acrylamide gel electrophoresis gives an estimated p*I* of 6.2, and only a single protein band was observed. A p*I* of 6.7 is predicted from the primary sequence of rhKDAO [31]. Previous investigations reported isoelectric points of 6.0 and 7.1 for the natural human DAO [32, 33].

SDS/PAGE of the purified enzyme gives a single protein band with an apparent molecular weight of 94 kDa (Fig. 1), although the primary sequence of rhKDAO predicts 83.4 kDa for the mature monomer. No corresponding band is detected in negative controls. The ~10 kDa discrepancy between the predicted and observed molecular weights suggests the expressed protein is substantially glycosylated (vide infra). Literature values for the apparent molecular weight of the natural human DAO have been reported as 70, 90, and 105 kDa by SDS/PAGE [33, 34, 35]. As evident in Fig. 1, the recombinant protein band represents the major protein in harvested media.

Several attempts to determine the native molecular weight by acrylamide gel electrophoresis were unsuccessful. Gel filtration on a calibrated AcA 34 Ultrogel column gave an apparent molecular weight of 113 kDa, unrealistically low for the homodimer (data not shown). Interestingly, Crabbe et al. [34] reported native molecular weights for the purified human placental DAO as 70 kDa by Sephadex G-200 gel filtration and 69.5 kDa by polyacrylamide gel electrophoresis. However, sedimentation equilibrium ultracentrifugation data in the same work indicated a native molecular weight of 235 kDa. Baylin and Margolis [35] reported the native molecular weight of human pregnancy plasma DAO as close to 200 kDa, as determined with Sephadex G-200.

Sedimentation equilibrium ultracentrifugation of rhKDAO gave an average apparent molecular weight of 210 kDa (five runs with two protein concentrations at two speeds, data not shown). Curvature of the A_{280} versus radius plot suggests the recombinant enzyme is in reversible equilibrium between dimers and higher-order complexes. The data could equally fit dimer-tetramer, dimer-hexamer, or dimer-octamer models, with an association constant of 1.2 ${A_{280}}^{-1}$ for the dimer-tetramer model.

Sedimentation velocity ultracentrifugation gave a sedimentation coefficient of 10 S and a diffusion coefficient of 4.4 Ficks (two runs, data not shown). The apparent molecular weight calculated by the Svedberg equation is 200 kDa. Both sedimentation velocity runs indicated a small shoulder of faster moving material, consistent with a very small amount of higher-order protein association.

Copper, calcium and TPQ

Copper, zinc, calcium, and magnesium content in the recombinant enzyme were investigated using either ICP emission spectroscopy or flame atomic absorption. Table 2 shows the results from three different enzyme preparations. The data suggest a mixture of copper and zinc occupying the active sites in the recombinant enzyme; the sum of copper and zinc approaches 2 mol per

Table 2. Metal ion and TPQ stoichiometry for rhKDAO as isolated from three enzyme purifications. Metal ion and TPQ values reported as mol per mol dimeric enzyme

Specific activity (IU mg^{-1})	Cu	Zn	Ca	Mg	TPQ
0.85	1.03	0.79	1.84	0.91	0.72
1.21	1.42[a]	ND[b]	ND	ND	1.04
1.25	1.48	0.40	3.20	0.0	1.08

[a]Value determined by flame atomic absorption spectroscopy; all other metal ion quantifications were by ICP emission spectroscopy
[b]Not determined

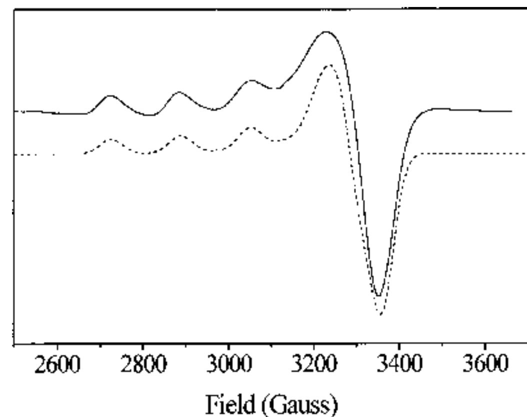


Fig. 2. X-band EPR spectra of the purified enzyme at ~200 μM in 100 M potassium phosphate buffer (*upper line*). The simulated spectra is shown with $g_{\perp} = 2.043$, $g_{||} = 2.265$, and $A_{||} = 162$ G (*lower dashed line*). Experimental conditions: 77 K, 9.42 GHz, 0.796 mW, 20 G modulation amplitude

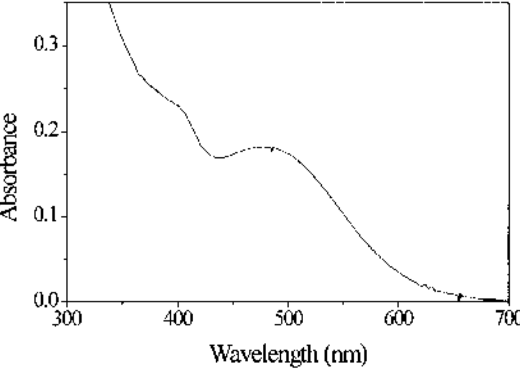


Fig. 3. Absorption spectra of the purified recombinant human kidney DAO, 16.4 mg mL^{-1} enzyme in 100 mM potassium phosphate buffer, pH 7.2

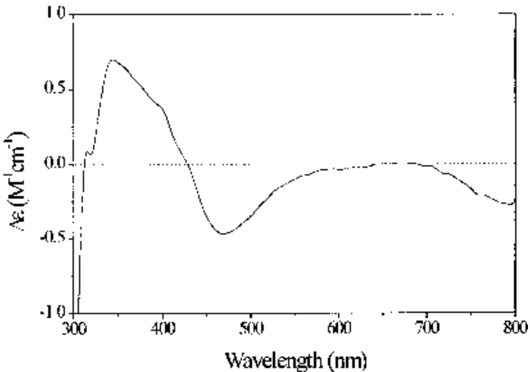


Fig. 4. Circular dichroism spectra of rhKDAO. Experimental conditions: 32 μM protein in 100 mM potassium phosphate buffer, pH 7.2, 1 nm resolution, 20 mdeg sensitivity, 1 s response, 500 μm slit, 5 accumulations

mol dimeric enzyme. In contrast, purified mammalian copper amine oxidases, as well as those from other organisms, are most often characterized as having two copper ions per dimer [1]. Our results indicate a correlation between copper content and specific activity for the recombinant enzyme; not surprisingly, samples with a higher copper content correspond to an increased specific activity.

Metal analysis and the lack of a manganese signal in the EPR spectra (Fig. 2) strongly suggest calcium occupies the putative second metal site in recombinant human kidney DAO. Purified enzyme was dialyzed either against metal-free 100 mM potassium phosphate buffer alone or against three changes of 2 L metal-free 100 mM potassium phosphate, 2 mM EDTA, pH 7.2, followed by extensive dialysis against metal-free phosphate buffer. The sample dialyzed against buffer alone was found by ICP emission spectroscopy to contain 2.37 mol calcium and 0.17 mol magnesium per mol of dimeric protein, whereas the sample treated with EDTA contained 2.16 mol calcium per homodimer, and magnesium levels were below the detection limit of the instrument.

Figure 2 shows the X-band EPR spectra of Cu(II) in purified rhKDAO. EPR parameters derived from simulated spectra ($g_{\perp} = 2.043$, $g_{||} = 2.265$, $A_{||} = 162$ G) are consistent with published values for CAOs from various sources [1]. Crabbe et al. [34] reported a $g_{\perp}$ value of 2.05 from Q-band EPR spectra of purified human placental DAO; however, the presence of manganese prevented the determination of other copper parameters. Manganese was not removed by passing the protein over a Chelex 100 column, leading those investigators to conclude the human placental DAO was a Cu(II)-Mn(II) metalloprotein, with an apparent stoichiometry of 2.0 mol copper and 2.4 mol manganese per mol enzyme dimer. The lack of a characteristic six-line Mn(II) signal in our spectra is convincing evidence against any manganese associated with the recombinant enzyme.

The purified protein is peach colored with a broad visible absorption band having a λ_{max} at 470 nm (Fig. 3). This spectral feature is associated with the TPQ and gives copper amine oxidases their recognizable color. The shoulder in the absorption spectra at 400 nm is an unidentified feature, perhaps due to a modified (possibly incompletely processed) form of the cofactor. The CD spectrum shown in Fig. 4 exhibits a negative band around 470 nm that is attributed to TPQ and

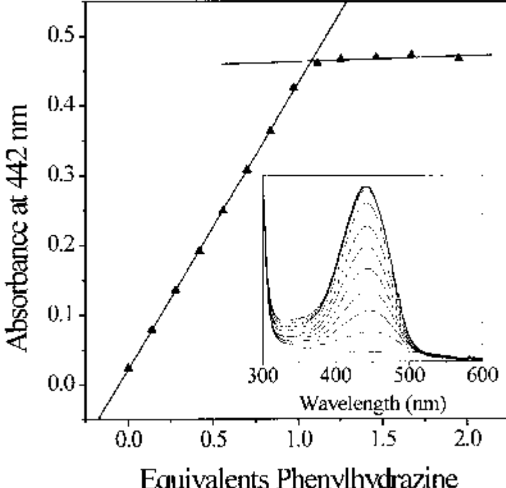


Fig. 5. Phenylhydrazine titration of TPQ in purified rhKDAO. Plot shows the change in absorbance at 442 nm versus equivalents phenylhydrazine added per enzyme dimer. One mL of enzyme (11.8 μM enzyme in 100 mM potassium phosphate buffer, pH 7.2) titrated with 2830 μM phenylhydrazine. The *inset* shows the increase in absoption of the intensely yellow-colored adduct with successive phenyhydrazine additions

a negative band around 800 nm from a Cu(II) d-d transition. These electronic transitions are consistent with other CAOs [1], and is consistent with a tetragonal Cu(II) complex with N,O donors.

Copper amine oxidases and the carbonyl reagent phenylhydrazine react to give a characteristic, yellow phenylhydrazone adduct that is commonly used to identify and quantify the TPQ cofactor [1]. Recombinant enzyme was titrated with phenylhydrazine and spectra were recorded after each addition when no further change in absorbance at 442 nm was noted. A typical titration demonstrating the formation of the intensely colored covalent adduct is shown in Fig. 5 and the results of TPQ quantification for three individual enzyme preparations are shown in Table 2. As described above for copper, the quantified organic cofactor correlates with specific activity.

The resonance Raman spectrum of phenylhydrazine-derivatized recombinant enzyme is shown in Fig. 6, along with that of the phenylhydrazone of TPQ-hydantoin, a model compound for the cofactor. Protein was concentrated after extensive buffer exchange to remove unreacted phenylhydrazine. The two spectra are essentially identical and thus conclusively identify TPQ as the quinone cofactor in the recombinant enzyme. Neither the visible absorption (Fig. 5) nor the resonance Raman spectrum of the phenylhydrazine adduct of rhKDAO suggests the absorption feature at ~400 nm (Fig. 3) reacts with this carbonyl reagent. Thus this unidentified absorption feature may be a modified form of the TPQ cofactor which is conformationally inaccessible to phenylhydrazine or one which is lacking a reactive carbonyl.

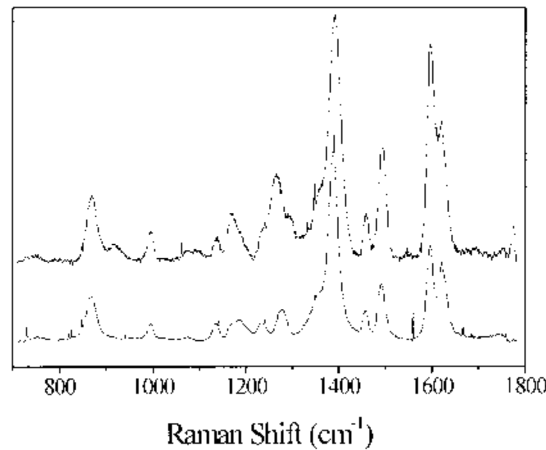


Fig. 6. Resonance Raman spectra of the phenyhydrazine-derivatized human rhKDAO (*upper spectrum*). The *lower spectrum* is the phenylhydrazine-derivatized model compound, TPQ-hydantoin. Experimental conditions: excitation 457.9 nm, power 40 mW, integration 1 min, 10 accumulations

Steady-state kinetics

K_M and k_{cat} were determined for several aliphatic diamines, the aromatic diamine DAB, and biologically important amines and polyamines. Initial rates were fit to the Michaelis-Menten equation and determined under the physiologically relevant conditions of 37 °C, pH 7.2, and an ionic strength of 150 mM. The results are summarized in Table 3.

Recombinant human DAO shows a substrate preference, as indicated by the specificity constant k_{cat}/K_M, for the diamines histamine (2-(4-imidazolyl)ethylamine) and 1-methylhistamine (1-methyl-4-[β-aminoethyl]imidazole), the metabolite of histamine *N*-methyltransferase (E.C.2.1.1.8). The aliphatic diamines putrescine and cadaverine give k_{cat}/K_M values close to, but lower than, those of the histidine metabolites. Histamine and 1-methylhistamine exhibit two of the three lowest turnover numbers, but note the turnover constants for all substrates investigated are quite similar and differ by less than a factor of 10. However, the determined K_M values vary by over three orders of magnitude, with the K_M for histamine about seven times less than that of any aliphatic diamine. The results of these experiments, namely that histamine and 1-methylhistamine are the preferred substrates, was completely unanticipated given the body of available literature, which inferred that the aliphatic diamines were the preferred substrates of human DAO (vide infra).

It should be kept in mind that the recombinant protein used for this work does not contain two copper ions and two organic cofactors per dimer; these experiments used rhKDAO with 1.42 copper ions and 1.04 titratable TPQ per dimer. Therefore the actual substrate turnover numbers for the natural hKDAO could be up to twice the values reported herein.

The pH dependence of k_{cat} for putrescine is shown in Fig. 7. The plot reveals a bell-shaped curve with

Table 3. Steady-state kinetic parameters and substrate specificity (k_{cat}/K_M) of rhKDAO. Errors are reported as standard error

Compound	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1}\ min^{-1}$)
Histamine[c]	2.8 ± 0.07	139 ± 0.6	50 ± 1
1-Methylhistamine	3.4 ± 0.3	103 ± 1.5	30 ± 3
Putrescine[c]	20 ± 1	475 ± 11	24 ± 1
Cadaverine	30 ± 2	453 ± 14	15 ± 1
DAB	110 ± 4	548 ± 4.6	5.0 ± 0.2
1,3-Diaminopropane	130 ± 10	487 ± 23	3.8 ± 0.3
1,6-Diaminohexane	150 ± 20	293 ± 14	2.0 ± 0.3
Ethylenediamine	630 ± 10	126 ± 0.70	0.20 ± 0.003
Spermidine	1100 ± 480	187 ± 0.4	0.17 ± 0.07
L-Lysine methyl ester	2800 ± 110	345 ± 4.6	0.12 ± 0.005
(–)-Arterenol	+ [a]	+	+
Benzylamine	+	+	+
3-Hydroxytyramine	+	+	+
Kynuramine	+	+	+
Spermine	+	+	+
5-Hydroxytryptamine	– [b]	–	–
L-Lysine	–	–	–
N^{α}-Acetyl-L-lysine methyl ester	–	–	–
Methylamine	–	–	–

[a]The positive symbol (+) denotes substrate oxidation could be detected, but at rates too low to determine kinetic parameters
[b]The negative symbol (–) indicates no rate was observed
[c]Partial substrate inhibition was observed only with histamine and putrescine

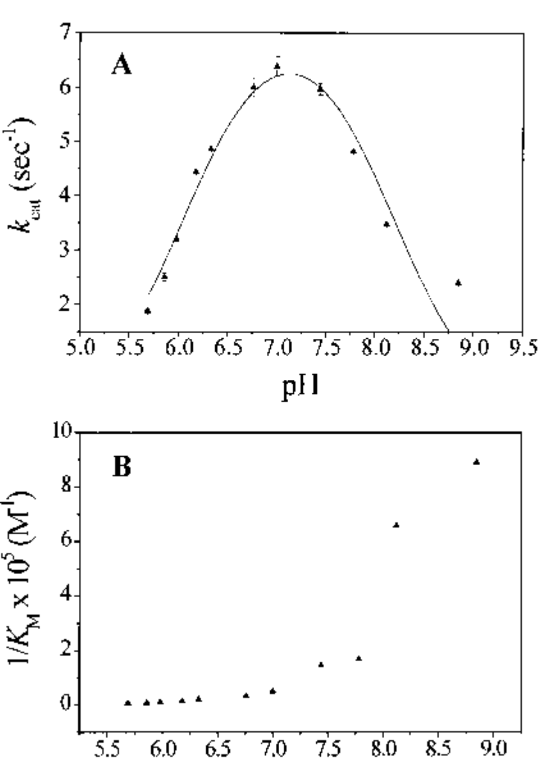


Fig. 7A, B. pH dependence of steady-state kinetic parameters for recombinant human kidney DAO. **A** k_{cat} versus pH. Fit to Eq. 4 gives two apparent pK_a values of 6.0 and 8.2. **B** $1/K_m$ versus pH

apparent pK_a values of 6.04 ± 0.06 and 8.22 ± 0.09. We attribute the lower pK_a to the ionization of the active site catalytic base *in the enzyme-substrate complex* (Asp373 in hKDAO). The higher pK_a likely represents the ionization of the product Schiff base (Scheme 1, species B, C). Klinman and co-workers [36, 37] have described detailed work on the pH dependence of steady-state parameters for the CAOs from bovine plasma and a yeast, and our data are consistent with their interpretation. A plot of $1/K_M$ versus pH reflects ionization potentials in the free enzyme and free substrate [29]. As shown in Fig. 7, the reciprocal Michaelis constant for rhKDAO is relatively constant from pH 5.7 to pH 7.0 and increases at higher pH values. These data could not be used to determine apparent pK_a values, although the data are in agreement with the interpretation of Klinman and co-workers of the catalytic aspartate residue in the free enzyme (Scheme 1, A) having an unusually high apparent pK_a (~8) and dramatically decreasing by about two orders of magnitude upon substrate binding.

Partial substrate inhibition was observed with histamine and putrescine (Fig. 8). Bardsley et al. [38] have previously described substrate inhibition with pig kidney DAO and attributed this inhibition as a primary kinetic salt effect, suggesting enzyme and substrate association might be partially rate limiting. Data for histamine and putrescine were fit to Eq. 3, which describes classical substrate inhibition where a second molecule of substrate binds to the enzyme-substrate complex to give a catalytically inactive complex. Panel B demonstrates that, at histamine concentrations of up to 250 μM, rhKDAO closely fits this model of substrate inhibition. However, at higher substrate concentrations (panel C) the data deviate from this model, suggesting that at higher histamine concentrations the enzyme kinetics are more complicated than the classical description of substrate inhibition. Figure 8C also shows the striking difference in kinetic behavior between histamine and 1-methylhistamine. Methylation of the imadazole

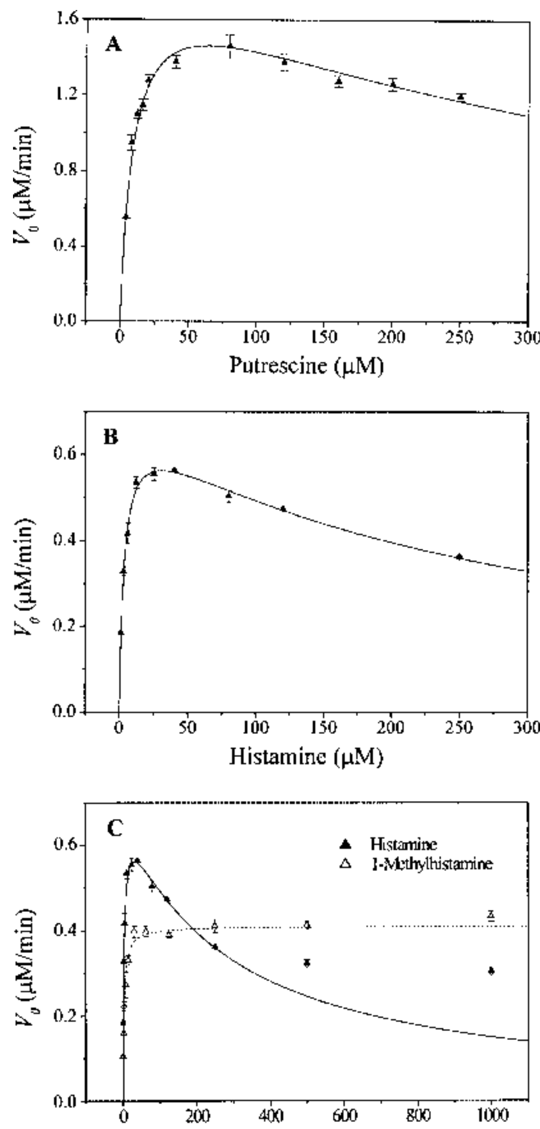


Fig. 8A–C. Plots of initial rates of reaction versus substrate concentration, demonstrating substrate inhibition observed with rhKDAO. Putrescine and histamine data were fit to Eq. 3; 1-methylhistamine data were fit to the Michaelis-Menten equation. **A** Putrescine substrate, $K_i = 430 \pm 40$ μM (0.8 μg enzyme at 1.00 IU mg^{-1} in a 1 mL assay volume, 50 mM HEPES, pH 7.5, ionic strength adjusted to 150 mM with KCl, at 37 °C). **B** Histamine substrate, $K_i = 280 \pm 30$ μM. **C** Comparison of histamine and 1-methylhistamine. Histamine and 1-*N*-methylhistamine assays used 7.3 μg enzyme (1.17 IU mg^{-1}) in a 1 mL assay volume, 50 mM HEPES, pH 7.2, ionic strength adjusted to 150 mM with KCl, at 37 °C

nitrogen apparently abolishes the substrate inhibition observed with histamine.

Two recent reports have suggested porcine kidney DAO is inhibited by heparin, either during prolonged binding on a heparin affinity column or through exposing the enzyme to free heparin [39, 40]. To investigate the possible effects of heparin on rhKDAO and to determine whether or not this is a general phenomenon of mammalian DAOs, the purified recombinant enzyme was incubated with an approximately two-fold excess of heparin over heparin-binding sites. K_M and k_{cat} values were not noticeably different from untreated protein for up to 72 h. Additionally, no correlation between specific activity and length of time the protein was bound to the heparin affinity column during purification was discerned.

Tissue-specific expression

A commercially available array of polyA$^+$ RNA representing 76 different human tissues and cell lines was probed with ^{35}P-labeled kidney DAO cDNA probes. The amount of polyA$^+$ RNA dotted on the nylon membrane was normalized by the manufacturer to give similar signal intensities for eight housekeeping genes and therefore varies from 53 to 780 ng. Each dot is 1 mm in diameter. Exposed and developed X-ray film is shown in Fig. 9. Although the data are not quantifiable, a qualitative interpretation is informative for comparative analysis of tissue-specific gene expression. The highest levels of DAO gene expression are in the placenta, followed by kidney tissue. Exposure at these two mRNA spots has clearly over-saturated the photographic media. Therefore actual mRNA levels may be orders of magnitude greater than that indicated by examination of the film. Gene expression is detectable in stomach tissue and quite significant through the distal tissues of the gastrointestinal tract. Prostate and liver tissues also show fairly strong signals. A positive can be seen with *E. coli* DNA; however, kidney DAO cDNA and the bacterial genome have no significant sequence homology, and this is assumed to be a false positive. A similar false positive has previously been reported [41]. Low, but detectable, positive results are seen in tissues from the stomach, bone marrow, lung, pancreas, testis, lymph node, and the parietal lobe and pons of the human brain. Negative results for controls other than the *E. coli* DNA, as well as for the majority of human tissues and cell lines, suggest that these are genuine positives. However, the weak results for these tissues, as compared to strong signals from other tissues, are such that actual gene expression levels in these tissues are normally quite low.

Discussion

Human kidney DAO, under the control of the metallothionein promoter, has been expressed in a *Drosophila* S2 polyclonal cell line. This work describes the first overexpression and purification of a copper-containing amine oxidase from any animal source. Expression levels for the secreted, recombinant enzyme are substantial, approaching 20 mg L^{-1} of cell culture. Almost certainly the abundant rhKDAO is a consequence of stably transfected *Drosophila* S2 cell lines generally containing multicopy genomic inserts of the

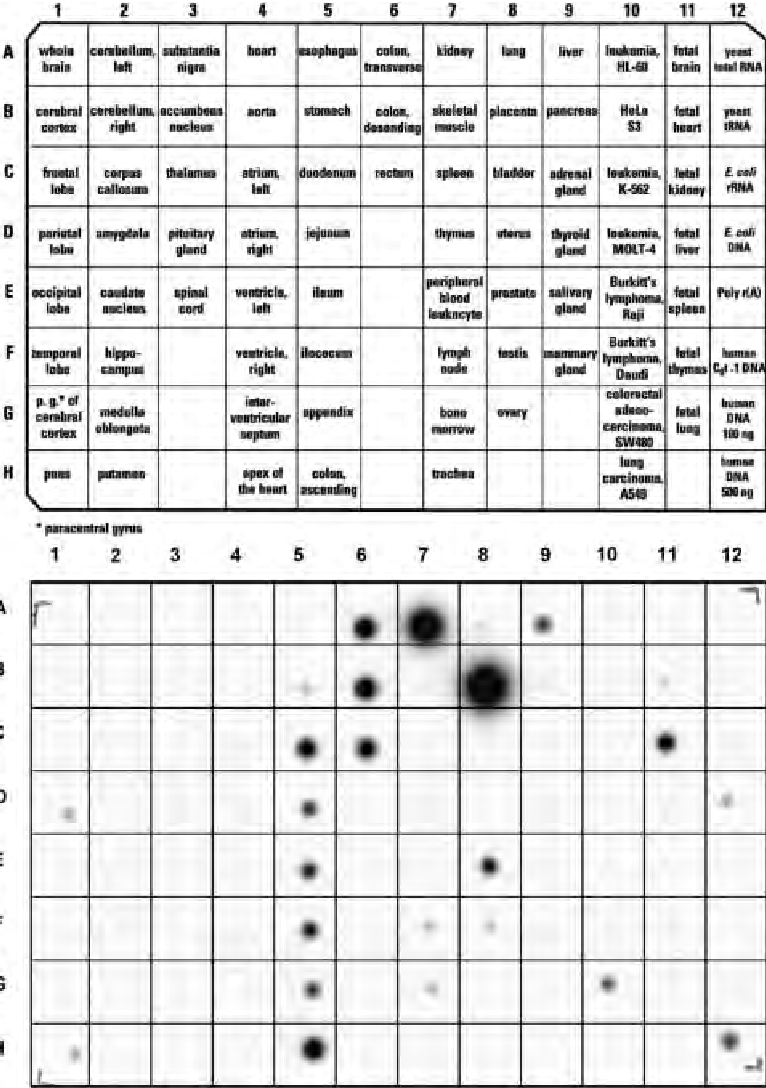


Fig. 9. Multiple human tissue expression array with polyA mRNA from 76 tissues and cell lines (Clontech). Probed with randomly primed ^{32}P-labeled oligonucleotides generated by PCR from a cDNA clone of human kidney DAO

expression and selection plasmids, often with 500–1000 copies arranged in a head-to-tail fashion [42]. Very recently, Koyanagi and co-workers [43] reported the heterologous overexpression of a copper amine oxidase from a multicellular organism, the plant *P. sativum*. Unfortunately, low expression levels and considerable clonal variation mar this notable accomplishment (Tanizawa K, personal communication). Expression of the *Arabidopsis thaliana* enzyme has previously been described in a baculovirus expression system [44]. The natural hKDAO heparin-binding site facilitated the development of a rapid purification scheme, resulting in the highly efficient recovery of >98% homogeneous protein. We present herein the first detailed spectroscopic investigation and characterization of a highly purified, recombinant human copper-containing amine oxidase.

Expression in serum-free media avoids introduction of exogenous copper amine oxidases [45, 46], as well as the considerable amount of undesired proteins naturally present in fetal calf serum. Furthermore, searches of the *D. melanogaster* genome have not revealed any sequences with significant homology to CAOs, nor, to the best of our knowledge, has a CAO been described from any insect source. However, one member of the copper-containing amine oxidase family, lysyl oxidase (E.C. 1.4.3.13), has been identified in the *Drosophila* genome [47]. Lysyl oxidase contains a lysine-tyrosylquinone cofactor and catalyzes the oxidative deamination of peptidyl lysine in elastin and collagen – a crucial step for connective tissue cross-linking [48]. Therefore the *Drosophila* Expression System, when used with serum-free media, is an excellent source of secreted human DAO, with the inherent advantages of low levels of unwanted proteins and apparently no contaminating CAOs, either exogenous or endogenous. *Drosophila* S2 cell have previously been used to express another copper enzyme, human dopamine *β*-hydroxylase, at protein yields quite similar to those reported in this work [49]. This expression system is thus a promising option for the convenient expression of other eukaryotic metalloenzymes.

Analytical ultracentrifugation results suggest rhKDAO contains between 20 and 26% glycosylation by weight, a value not unreasonable for the natural mammalian enzyme. The results given by Crabbe et al. [34] indicate approximately 40% glycosylation for the natural human placental enzyme. Gel filtration by Baylin and Margolis [35] suggest 20% glycosylation for the natural human pregnancy plasma DAO. For comparison, the porcine kidney DAO has been estimated at 20% and 11% carbohydrate by weight [50, 51]. Although the data on glycosylation in mammalian DAOs are limited – and it must be noted that differences in glycosylation could very well be tissue-specific – the extent of glycosylation by weight in rhKDAO is within the range of values reported for the natural enzymes.

SDS/PAGE and analytical ultracentrifugation results are consistent with a substantially glycosylated recombinant enzyme, as would be expected for a protein with potential glycosylation sites and processed through the secretory pathway. Electrospray mass spectrometry of the recombinant human kidney protein gave a large m/z "hump" with no resolvable peaks, indicative of a population of heterogeneously glycosylated proteins (data not shown). Four consensus *N*-glycosylation sequences (NXS/T) are present in the peptide sequence of human kidney DAO. *N*-Linked glycosylation in *Drosophila* is much like that in other insect cells, with a high mannose content, although *O*-linked glycosylation is also possible [52, 53]. Glycosylation of the recombinant enzyme is expected to be different from the enzymes processed in mammalian cells, and strategies to reduce the extent of glycosylation in rhKDAO are actively being pursued.

The heterologously expressed enzyme contains substoichiometric copper and displays some variability in copper levels from different purifications. The data shown in Table 2 indicate the copper content ranges from 1 to 1.5 ions per dimer. The recombinant enzyme most likely contains tightly bound zinc in the remaining active sites; copper and zinc together account for nearly full metal occupancy of the two active sites in each protein homodimer. Zinc has previously been described in recombinant *H. polymorpha* CAO overexpressed in *Saccharomyces cerevisiae*, and zinc was determined to have a high affinity for the active site and to compete with copper for binding [54]. The crystal structure of a zinc-substituted *H. polymorpha* enzyme has recently been solved and reveals zinc in the active site, coordinated by three histidine residues and a tyrosine residue (copper ligands in the active enzyme and the tyrosine precursor of TPQ) [55]. These residues are remarkably similar in position to those described in the active site of apoCAO from *Arthrobacter globiformis* [15].

We believe active-site metal incorporation most likely occurs intracellularly during rhKDAO processing in the *Drosophila* secretory pathway, and not following secretion into media loaded with 500 μM copper, in which case fully copper-loaded protein could be expected. Rae et al. [56] have made a convincing argument for extraordinarily low levels of "free" intracellular copper, less than one per cell, and suggested copper-dependent enzymes require accessory factors (i.e. metallochaperones). However, their analysis is based upon *cytoplasmic* superoxide dismutase and would not necessarily apply to the contents of membrane-bound organelles, such as those of the secretory pathway. Copper most likely enters the *Drosophila* secretory pathway via a P-type ATPase, homologous to the human Menkes disease protein, localized in the trans-Golgi network [57]. To date, no accessory protein for any CAO has been described. Furthermore, *Drosophila* S2 culture is quite likely a zero background expression host and therefore would not be expected to possess a specific CAO metallochaperone. Copper and zinc content in the overexpressed enzyme may be determined by the total metal ion availability in the secretory pathway and the relative affinity of the active site for these two transition metals. Differences in metal loading between purifications might be the consequence of subtle and as yet unrecognized factors during cell culturing and protein expression.

This study indicates calcium occupies the putative second metal binding site in recombinant human kidney DAO. The high specificity and affinity of this site for calcium, supported by the resistance to removal by EDTA ($\log K = 10.61$ at 25 °C [58]), strongly suggest calcium would be present in the natural enzyme. Stoichiometric calcium is also present in highly purified equine plasma copper amine oxidase (unpublished results).

Although calcium sites in proteins vary considerably with regard to their coordination structure, the second-metal site in copper amine oxidases is unusual when compared to other structurally defined sites (see [59] for a recent review of the diverse calcium binding sites in proteins). Five putative KDAO calcium ligands can be deduced from sequence alignment and the crystal structures of the CAOs from *E. coli* and *P. sativum* [13, 14]. Three of these are aspartyl carboxylates and the backbone carbonyl of a leucine in the sequence AspLeuAsp. These residues are located at the C-terminus of one β strand. The other two protein-derived oxygen donors are an aspartyl carboxylate and an adjacent backbone carbonyl (Leu), located at the N-terminus of another β strand. Interestingly, the opposite ends of these two β strands (7–10 residues away) also provide the active site copper ligands. One coordinating water molecule is resolved in the crystal structures, giving an octahedral coordination geometry with calcium-oxygen distances from 2.2 to 2.5 Å. Most calcium ions in biology are heptacoordinate and associated with helical and loop regions [60]. The well-known and extensive family of helix-loop-helix (EF hand) calcium sites is typically seven-coordinate with three carboxylates (one of which is bidentate), two carbonyls, and one water molecule in a pentagonal bipyramid. EF hand ligands are all located in a short sequence at the end of one helix, a small loop, and the beginning of another helix. In contrast, the calcium binding residues in CAOs are at the ends of two

β strands, separated in sequence by more than 130 intervening amino acids.

Calcium ions are critical for a variety of important biological processes, including some involving extracellular proteins [61, 62, 63, 64, 65, 66]. The function of the second metal site in copper-containing amine oxidases remains to be elucidated. However, the bound calcium ion likely serves to stabilize the structure of this extracellular enzyme, although a role in modulating activity is also possible but unlikely, given the apparently high affinity for calcium and the separation between the putative Ca site and the active site.

Recombinant human kidney DAO is conclusively demonstrated to possess the TPQ cofactor. Visible absorption, CD, titration with phenylhydrazine, and resonance Raman of the phenylhydrazine-derivatized enzyme are entirely consistent with previously characterized copper amine oxidases from various sources [1, 67]. Similar correlations between specific activity and titratable TPQ have been described by Klinman and co-workers [23, 68] for the bovine plasma amine oxidase and the recombinant yeast methylamine oxidase. As shown in Table 2, the titratable TPQ from three different preparations is consistently around 72% of that which could be expected given the copper to protein stoichiometry, assuming that each active site containing copper would readily convert the precursor tyrosine to TPQ. It is presently unclear whether low TPQ-to-copper ratios reflect incomplete organic cofactor biogenesis or enzyme conformations in which the quinone cofactor is inaccessible to phenylhydrazine. Low and variable TPQ content is not uncommon in CAOs [1]. For example, recombinant yeast methylamine oxidase has been described as containing 2.0 mol copper but only 1.5 mol phenylhydrazine-titratable TPQ per mol of enzyme dimer [68], a TPQ to copper ratio strikingly close to the results presented herein.

Recombinant human kidney DAO shows a clear substrate preference for diamines, with the highest specificity constants (k_{cat}/K_M) for histamine and 1-methylhistamine. It is also apparent that substrate specificity depends on the separation between the substrate's two amine groups, as well as other structural features. Although diaminopropane is a good substrate, the polyamine spermine, also with three methylene carbons between a primary amine nitrogen and the closest secondary amine, is a very poor substrate, with an oxidation rate nearly undetectable using our assay conditions. Spermidine, on the other hand, has four methylene carbons between one of the primary amines and the secondary amine and is a fair substrate, but with a specificity constant two orders of magnitude less than that for putrescine. L-Lysine methyl ester is a poor substrate, and no oxidation was observed with either L-lysine or N^{α}-acetyl-L-lysine methyl ester. Therefore, it is extremely unlikely that the DAO-catalyzed oxidation of peptidyl lysines would occur to a physiologically relevant extent in vivo.

Earlier literature reported the substrate preference of DAO solely determined by relative rates of oxidation, a necessary criterion given the limited amounts of enzyme available. Misleading conclusions regarding substrate preference were inevitable, given the difficulty in obtaining highly purified mammalian DAO, partial substrate inhibition, and the millimolar substrate concentrations used. Generally, cadaverine was reported as the substrate with highest rate of oxidation, up to nearly 1.5 times the rate for putrescine. Histamine was described as having only 17–50% the relative rate of oxidation as compared to putrescine [32, 34, 69]. Unfortunately, this has resulted in a predisposition to downplay or discount a possible role for DAO in histamine metabolism in much of the earlier literature.

Our work establishes a K_M for histamine equal to 2.8 μM and a k_{cat}/K_M of 50 $\mu M^{-1} min^{-1}$. Previously reported K_M values for DAO purified from human tissues range from 1.5 μM for the enzyme isolated from pregnancy serum to 19 μM for the intestinal DAO [30, 70, 71]. The apparent K_M determined for 1-methylhistamine is 3.4 μM, whereas earlier work reported 97 μM [30, 71]. It is interesting to note that aliphatic diamines and polyamines would be protonated at physiological pH and therefore be di- or polycationic. Histamine, however, would only be about 8% present as the doubly charged species at pH 7.2.

The recombinant enzyme exhibits an apparent K_M for putrescine of 20 μM. Previously reported values from purified human DAOs range from 13 μM to 83 μM [30, 71, 72]. Hölttä et al. [72] reported that human seminal plasma DAO displays a K_M of 100 μM for spermine and 560 μM for spermidine. In contrast, we find an apparent K_M of 1.1 mM for spermidine, and that of spermine could not be determined with our experimental conditions. This discrepancy is perhaps due to contaminating enzymes in the seminal plasma enzyme purification or impurities in commercially available polyamines; in fact, Hölttä and co-workers described contaminating spermidine and putrescine in purchased spermine.

Scheme 1 diagrams the proposed steps in the catalytic cycle of CAOs [73, 74]. Detailed kinetic studies have elucidated many of the details of the reductive half-reaction. Proton abstraction from the substrate Schiff base (species B) has been shown to at least partially contribute to the rate-determining step in some mammalian CAOs [36, 75]. Substrate turnover (k_{cat}) values for rhKDAO are quite similar among the substrates investigated in this work, from 1 to 10 s^{-1}, suggesting a common rate-limiting step.

The oxidative-half reaction is less well understood. Anaerobic substrate-reduced enzyme has been shown to be a kinetically competent, equilibrium mixture of the Cu(II)-aminoquinol and Cu(I)-aminosemiquinone radical (species D, E), and substantial chemical precedence suggests the Cu(I)-aminosemiquinone species would readily react with dioxygen [76, 77, 78]. However, an obligatory role for Cu(I) has not been established, and a

viable mechanistic alternative that does not invoke a change in the oxidation state of copper has been proposed [79, 80]. Copper amine oxidases from different sources have been suggested to use distinctly different mechanisms for reoxidation [81]. We anticipate that the work presented herein will facilitate experimental design to probe the molecular details of TPQ reoxidation and the concomitant reduction of dioxygen in the human enzyme.

Biological implications

Mammalian DAOs are found in several tissues, with the highest activities described in placenta, small intestine, and kidney, but they are also present in liver, lung, fibroblasts, salivary glands, and seminal fluid. Low basal levels of DAO are present in human serum and are believed to derive from the gastrointestinal tract [1]. However, the circulatory DAO activity increases significantly following the intravenous injection of heparin and during pregnancy [82, 83, 84, 85, 86].

The human expression array results presented herein largely concurs with earlier studies describing the tissues with highest DAO activity, but some of the positive results were unanticipated. To the best of our knowledge, DAO activity has not been reported in lymph nodes or bone marrow, and the expression array indicates positive, albeit low, DAO mRNA in two specific regions of the human brain, the parietal lobe and the pons. It will be of considerable interest to see if future studies support the apparent localized expression of DAO in the human brain. DAO activity in this organ is normally extremely low [87, 88]. However, evidence exists for increased DAO activity in rat brain following focal brain injury, an increase that tracks the rise in putrescine concentration [89]. DAO is generally not thought to play a role in histamine metabolism in the brain, where histamine serves as a neurotransmitter. The physiological function (or functions) of this enzyme in the human brain remains to be demonstrated.

Several investigations have reported putrescine (1,4-diaminobutane) or cadaverine (1,5-diaminopentane) as the substrate with the highest rate of oxidation and suggested these are the preferred substrates for mammalian DAOs [30, 32, 34, 72, 90, 91]. Putrescine deamination is the rate-limiting step in catabolism of the polyamines spermidine and spermine [92]. These polyamines are found in millimolar concentrations in cells and in seminal fluid and are important in cell proliferation and differentiation [1, 93, 94]. Given the steady-state parameters determined in this work, putrescine and spermidine could be viable substrates for the DAO known to be present extracellularly (e.g., recall that the expression array gives a strong positive for the prostrate). Additionally, γ-aminobutyraldehyde, the product of putrescine oxidation by DAO, is a precursor for the neurotransmitter γ-aminobutyric acid in some tissues [95].

The current work, however, indicates a substrate preference for histamine and 1-methylhistamine, suggesting human DAO may play an important role in histamine metabolism. Histamine is a potent pharmacological agent with profound biological effects, including smooth muscle contraction, vasodilation, allergic response, gastric acid secretion, and stimulation of adenylate cyclase activity in neurons, all of which are mediated by specific G-protein-coupled receptors [96].

Historically, DAO has been proposed to function as a safeguard against deleterious effects from exogenous amines. In the intestinal tract this enzyme is thought to detoxify ingested histamine [30, 97] and in placental tissues is suggested to protect from high levels of fetal putrescine and histamine [98, 99]. However, the striking substrate inhibition observed with histamine seems to make DAO an unlikely choice to serve as the first line of defense against toxic levels of histamine. In circulation, histamine concentrations are usually only a few micromolar, a physiologically relevant condition in which DAO could be very efficient in removing undesired histamine. Baenziger, Haddock and co-workers [45] have demonstrated DAO activity enhances histamine metabolism and uptake by cultured vascular endothelial cells and have proposed that DAO in circulation can bind to specific endothelial cell surface receptors and participate in the sequential, enzymatic degradation of histamine. Collectively, the available evidence suggests that DAO may be involved in several critical biological processes in mammals, perhaps depending in part on spatial and temporal factors, as well as the level of expression.

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Diamine Oxidase Activity

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Review

Advances in the Clinical Application of Histamine and Diamine Oxidase (DAO) Activity: A Review

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Abstract: The serum level of diamine oxidase (DAO) reflects the integrity and maturation of the small intestinal mucosa. This measure is important in diagnosing various diseases, including chronic urticaria tachyphylaxis, multiple organ dysfunction syndrome, preterm abortion, and migraine. This review aimed to summarize the findings of previous studies on the changes in DAO levels in diverse diseases and the application of this enzyme in the clinical setting, as well as the roles of this enzyme under physiological and pathological conditions. The advances in the mechanism and clinical application of DAO presented in this review will contribute to a better understanding of this enzyme and open up new and broader perspectives for future basic research and clinical applications.

Keywords: histamine; diamine oxidase; molecular mechanisms; clinical application

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1. Introduction

Diamine oxidase (DAO) is a secretory protein located in the cytoplasm of human and mammalian upper intestinal mucosa chromaffin cells and is responsible for catabolizing histamine (mainly extracellular) to stop allergic reactions [1]. DAO level is extremely low in peripheral blood; this enzyme is mainly distributed in the intestine and abundant in the kidney, placenta, and other organs [2]. With the maturation of the intestinal mucosa, baseline levels of DAO increase significantly, particularly in the small intestine. Various factors, including drugs and food, influence DAO levels [3].

DAO level in peripheral blood is comparatively stable, whereas the level in the intestinal mucosa decreases during ischemia, hypoxia, or nutritional dysfunction of the intestinal luminal tissues, consequently decreasing DAO level in the blood. DAO level reflects intestinal damage and repair. Specifically, plasma DAO levels can be used to monitor the function of the small intestinal mucosal barrier under noninvasive conditions. Thus, this measure has gained increasing clinical attention in recent years in the diagnosis of gastrointestinal diseases, histamine intolerance (HIT), migraine, and abnormal pregnancy and the prognosis of tumors.

Histamine is mainly produced by mast cells, platelets, basophils, histaminergic neurons, and enterochromatin cells, where it is stored in vesicles and released in response to stimulation. It is synthesized from the amino acid histidine by pyridoxal phosphate-containing L-histidine decarboxylase (HDC) and functions by binding to four receptors on target cells in various tissues. It causes smooth muscle cell contraction, vasodilation, increased vascular permeability and mucus secretion, tachycardia, arrhythmias, altered blood pressure, stimulated gastric acid secretion, and nociceptive nerve fibers [3].

Histamine is primarily metabolized in two ways: oxidative deamination by DAO and cyclomethylation by histamine-N-methyltransferase (HNMT). Histamine localization

determines whether it is catabolized by DAO or HNMT. DAO is stored in peripheral tissues, such as the kidney, colon, placenta, and thymus; it is responsible for scavenging extracellular histamine after the release of mediators and is secreted into the circulation when stimulated [4]. DAO can also catabolize other polyamines, such as putrescine and spermidine. By contrast, HNMT is a cell membrane protein that can only transform histamine into the intracellular space (containing mast cells and histaminergic neurons).

In recent decades, serum DAO has gained considerable interest from researchers because of its role in diagnosing various diseases. However, past research on DAO has mostly focused on fundamental experiments and there is a lack of publications discussing the latest developments in the application of DAO in clinical practice. This review aims to elucidate the differential expression of DAO in diverse diseases (Table 1) and the application of this enzyme in the clinical setting.

2. The Role of DAO Activity in the Monitoring of Diverse Diseases

2.1. Role of DAO in Detecting Various Gastrointestinal Diseases

Several human and animal experiments have confirmed that plasma DAO level is a key marker for assessing the function of the intestinal mucosal barrier [5]. The crucial role of DAO level in monitoring intestinal mucosal damage has also been validated in various clinical scenarios [6–9]. Injury to the intestinal mucosal barrier increases epithelial cell permeability, which consequently triggers an inflammatory response that is closely associated with inflammatory bowel diseases, especially Crohn's disease [10,11]. Elevated plasma DAO level indicates the repair of intestinal damage, and it can be used as a sensitive and precise marker in monitoring Crohn's disease activity [12,13]. DAO is a useful molecular parameter for the early and accurate diagnosis and identification of small bowel obstruction. Clinically, an increase in serum DAO level (twice the basal level) may be a useful marker to diagnose simple to strangulated intestinal obstruction. An animal study showed that serum DAO level significantly increases in simple intestinal obstruction but progressively decreases in strangulated intestinal obstruction [14]. This result can be attributed to reduced blood flow caused by strangulated intestinal obstruction. Additionally, an increasing number of animal experiments have demonstrated the value of DAO in the early diagnosis of acute mesenteric ischemia and superior mesenteric artery occlusion [15,16]. Another study specified that DAO = 29.81 U/L can be used as an early diagnostic criterion for diagnosing superior mesenteric artery occlusion [17]; however, this criterion is insufficient for clinical diagnosis, and extensive clinical studies in humans are needed.

2.2. DAO and Migraine

Migraine is a common neurological disorder and the third most common disorder, affecting up to 1 billion people worldwide [18,19]. HIT arises from a deficiency of DAO, and headache is one of the most documented of the several multifaceted symptoms associated with HIT. In a single clinical study [20], 198 volunteers were divided into migraine and control groups, and DAO level was measured using ELISA. The mean DAO level was significantly lower in patients with migraine than in the healthy volunteers. Moreover, DAO deficiency was more prevalent in patients with migraine (87%) than in healthy volunteers. Another RCT by Joan et al. [21] verified that 1 month of oral DAO enzyme supplementation reduces pain duration by 1.4 h in patients with episodic migraine; however, this treatment exerts no significant effect on migraine attack frequency or pain intensity.

Regarding the mechanisms underlying the association between DAO and migraine, a recent genetic study of 22 genome-wide association studies found that 38 genes are susceptibility loci for migraine [22]. Another study reported that the frequency of mutations caused by C2029G DAO single-nucleotide polymorphisms (SNPs) is significantly higher in patients with migraine than in healthy controls and that the C314T mutant allele of HNMT and the C2029G polymorphism of DAO interact to increase the risk and impact of migraine [23]. Another study examining alleles and the frequency of their allelic variants

in patients with migraine found that the *DAO* SNP rs10156191 is associated with reduced DAO activity and is related to the risk of migraine onset, particularly in women [24]. These results suggest that the risk of migraine is associated with the SNP rs10156191 and sex.

However, a recent study exploring the relationship between serum DAO and histamine levels and three polymorphisms in the *DAO* gene (rs10156191, rs1049742, and rs1049793) found similar frequencies of *DAO* genes and allelic variants in patients with migraine and controls [25]. Surprisingly, serum DAO levels were significantly higher in the patients with migraine than in the controls. The opposite conclusion may stem from differences in the methods used to measure DAO level, whether age- and sex-matched subgroups were used, and variations in the inclusion criteria.

2.3. DAO in Pregnancy Monitoring

DAO can be generated in large quantities by the placenta and is thought to be a paracrine signal during endometrial shedding and embryonic implantation [26], serving as a metabolic barrier against the excessive passage of active histamine from the placenta into the maternal or fetal circulation [27]. The balance between histamine and its degrading enzyme DAO plays an essential role in pregnancy [28]. Serum DAO activity has significant sex differences, with females showing greater fluctuations in serum DAO levels than males [29]. Hamada et al. [30] found that serum DAO levels vary with the menstrual cycle, with markedly lower plasma DAO levels in the follicular phase than in the luteal phase. DAO is synthesized by placental and trophoblast cells, explaining the high plasma DAO level during pregnancy [31]. The maternal plasma DAO level exponentially increases during the first 20 weeks of pregnancy by up to even 1000 times the pre-gestational level [28], which consequently decreases plasma and urinary histamine levels in the maternal circulation. In abnormal pregnancies, compared to normal pregnancies, the maternal plasma DAO level ceases to rise and the circulating histamine level increases, which significantly increases the risk of threatened abortion, pre-eclampsia, and spontaneous abortion [31–33]. Moreover, this level drops to pre-pregnancy values within 10–15 days after delivery [34]. Low DAO levels can also be used as a diagnostic tool for trophoblastic diseases. In pregnant females with trophoblastic diseases, such as choriocarcinoma and hydatid mole, DAO levels remain low despite high titers of human chorionic gonadotropin. In molar pregnancies, DAO levels are equal to those in normal pregnancies in early gestation but decline after 15 weeks of gestation. In addition, a sharp drop in the DAO curve is a sign of fetal distress or intrauterine death [27].

2.4. DAO as a Predictor of the Gastrointestinal (GI) Tract Toxicity of Drugs

In rats, the DAO level in blood significantly correlates with the level in small intestinal mucosal villi and with the severity of intestinal toxicity caused by anticancer drugs such as 5-fluorouracil (FT) [35]. Tsutomu et al. [36] measured serum DAO levels in 20 patients with gastric cancer during adjuvant chemotherapy with oral FT anticancer drugs and found that antitumor drug treatment decreased DAO levels and health status positively correlated with DAO levels in these patients. A recent prospective cohort study involving 50 patients with esophageal cancer treated with docetaxel + cisplatin + 5-FT reported that plasma DAO level reflects the ability of the intestine to absorb amino acids and thus can be used as an indicator of the efficacy of chemotherapy in patients with esophageal cancer [37]. Serum DAO activity decreases gradually over the course of anticancer drug treatment, and the percentage decrease in DAO activity correlates closely with the severity of gastrointestinal toxicity [38]. This result indicates that plasma DAO levels can be used to monitor and evaluate the GI toxicity response to chemotherapy in patients with cancer [38,39]. Colchicine increases gut permeability, alters the intestinal microbiota, exacerbates flora displacement, and inhibits inflammatory response in mice, which may increase toxic load in the mouse intestine. Thus, the serum levels of DAO and lipopolysaccharide are increased [40]. Plasma DAO level is an important measure in conducting drug/food therapy studies of anticancer drug-induced

gastrointestinal toxicity and monitoring intestinal integrity and related complications in gastric, esophageal, liver, colorectal, breast, head, and neck cancers [41–46].

Table 1. Serum DAO in different diseases.

Disease	Origin	Change of Serum DAO *	Comments	Refs.
gastrointestinal diseases	rats, mice, humans	increased	More clinical studies are needed to support the diagnostic value of DAO.	[12,15–17]
migraine	humans	decreased/increased	Oral DAO supplementation might be a new therapy for migraines.	[18–21]
pregnancy	humans	increased	Serum DAO has a role in pregnancy confirmation and screening for trophoblast diseases.	[23,25,26]
gastrointestinal tract toxicity	humans, rats, mice	decreased/increased	The precision of DAO levels reflecting toxicity needs further study.	[28–31]
liver disease	humans	increased	DAO might be a potential biomarker in patients with liver and intestinal dysfunction.	[32,33,35]
histamine intolerance	humans	increased	The role of DAO in the diagnosis of histamine intolerance and its treatment has been clarified.	[38–41]

* DAO expressed differently in a variety of diseases, suggesting the respective direction of future research.

2.5. DAO in Hepatitis and Post-Hepatitis Cirrhosis

Plasma DAO level has also been associated with the development and prognosis of many liver disorders, such as hepatitis, cirrhosis, and orthotopic liver transplantation. Li et al. [47] conducted a 1-month follow-up study of 106 patients newly diagnosed with acute-on-chronic hepatitis B liver failure (ACHBLF) and found that plasma DAO level reflects the severity of ACHBLF and is an independent risk factor for 1-month mortality. Furthermore, DAO level is more sensitive than the conventional model for end-stage liver disease, with a plasma DAO level of 15.2 ng/mL as the cut-off point. As for patients with hepatitis B virus-associated decompensated cirrhosis, plasma DAO levels > 19.7 ng/mL have been associated with high 6-month readmission rates [48]. Considering the connection between DAO level and intestinal mucosal condition, a previous study measured serum DAO and endotoxin levels in patients with liver cirrhosis and found that DAO level is significantly higher in patients with liver cirrhosis than in healthy individuals [49]. This result suggests that DAO level is a sensitive marker for the early diagnosis of gut failure in liver cirrhosis.

Recent studies on serum DAO levels have extended their focus to liver transplantation. The only curative procedure for patients with end-stage liver disease is orthotopic liver transplantation (OLT). To assess the role of histamine and plasma DAO in OLT, an RCT of 22 liver transplant patients and 22 healthy adults as controls found that the baseline levels of histamine and plasma DAO were markedly elevated in patients undergoing OLT; however, the concentration of histamine decreased and DAO increased significantly during OLT [50]. This result can be attributed to the intraoperative use of norepinephrine. As mentioned in these studies, serum DAO mainly reflects the function of the intestine rather than the liver.

2.6. DAO and HIT

HIT is a disturbance in histamine homeostasis caused by reduced intestinal degradation of histamine due to DAO deficiency [51]. DAO deficiency may be a major cause of HIT, in which alterations in histamine homeostasis lead to a decrease in intestinal degradation and a subsequent increase in plasma [52]. DAO deficiency may be congenitally caused by genetic mutations in DAO genes or alterations in protein coding resulting in decreased DAO levels; it may also be acquired from lesions that reduce DAO secretion, particularly

in inflammatory or degenerative bowel diseases [53]. The multifaceted clinical symptoms associated with HIT include headache, gastrointestinal disturbances (such as abdominal pain, diarrhea, and flatulence), urticaria, pruritus, nausea and sneezing, runny nose, cardiac arrhythmias, hypotension, and muscle pain [21].

HIT is a disease characterized by a disequilibrium between accumulated histamine and histamine degradability. The degradation of histamine in the intestine decreases with reduced DAO activity, resulting in the accumulation of histamine in the blood plasma and adverse reactions [54,55], which mainly originate in the gut [56]. Impaired histamine degradation due to reduced DAO activity and subsequent histamine overload may lead to symptoms similar to those of allergic reactions [3]. In patients with HIT, ingestion of histamine-rich foods, alcohol, or drugs that either release histamine or block DAO may induce diarrhea, headache, respiratory allergies (e.g., atopic asthma and allergic rhinitis) [57], hypotension, cardiac arrhythmias, urticaria [58], pruritus, flushing, and other conditions. This finding indicates that serum DAO level is valuable in the diagnosis of HIT [59], and symptom severity is associated with DAO deficiency.

A recent questionnaire follow-up study of 133 outpatients with HIT (serum DAO values < 10 U/mL) with onset symptoms (primarily gastrointestinal, cardiovascular, respiratory, and skin complaints) was conducted to explore the onset of non-specific GI and extraintestinal symptoms arising from the distribution of the four histamine receptors in different organs and tissues of the body. Results of this study showed that patients with HIT predominantly had gastrointestinal manifestations, with abdominal distention being the most common symptom (92%), followed by cardiovascular symptoms and, finally, respiratory and skin complaints [3,60–62]. Another study showed that in patients with low DAO levels (<40 HDU/mL), the clinical symptoms typical of HIT disappear, and serum DAO levels significantly increase after the introduction of a histamine-free diet [63]. Similar effects can be exerted by oral DAO supplementation in patients with HIT [64]. Cucca et al. found that patients with DAO values of 3–10 U/mL exhibit the most complicated clinical presentation but also respond best to treatment with a low-histamine diet and/or DAO supplementation [65]. The prevailing consensus is that incorporating a histamine-reduced diet and/or oral microbial DAO capsules into a targeted dietary intervention can help alleviate HIT-related symptoms [66,67]. HIT has been a hot research topic for almost a decade, but evidence-based, double-blind, placebo-controlled, and crossover in vivo studies are still necessary to understand the effects of oral DAO supplementation and provide a basis for further investigations on HIT.

3. Effect of Food/Drugs on DAO Determination

Histamine-induced food intolerance used to be defined as an intolerance to red wine and the symptoms of allergy after ingestion of histamine-rich foods, which is not IgE-mediated. These symptoms are milder and less persistent, with a deficiency of diamine oxidase leading to a reduction in histamine degradation that results in excessive accumulation of histamine [68], thus explaining the alleviation of HIT symptoms after a histamine-free diet or antihistamines [69].

A decrease in intestinal mucosal DAO activity was found in patients with food allergy [70]. Foods that are known to contain histamine include milk, eggs, tuna, sauerkraut, cheese, nuts, seafood, fresh fruits, and vegetables associated with pollen, all of which are common allergens [68,71–73].

Xu et al. observed in a mouse model of induced inflammatory bowel disease that polysaccharides of Tremella fuciformis could restore intestinal microbiota and microbial metabolites and then significantly increase intestinal flora diversity, thereby reducing serum DAO activity; this suggests that Tremella fuciformis may serve as a food supplement to improve intestinal disease [74].

As mentioned before, serum DAO activity varies more in females, and serum DAO values vary with the menstrual cycle, peaking at the luteal phase [75]. Miyoshi et al. analyzed the relationship between DAO activity and dietary nutrient intake/energy ratio

through dietary surveys and measurement of serum DAO activity in 34 healthy Japanese women during the follicular and luteal phases and found that serum DAO activity was positively correlated with the intake of long-chain fatty acids, especially saturated and monounsaturated fatty acids; however, it was unrelated to consumption of medium and short-chain fatty acids, protein, carbohydrate lipids, and dietary fiber. Further studies found that serum DAO activity in the luteal phase was also positively correlated with dietary intake of phosphorus, calcium, magnesium, iron, zinc, and vitamin B_{12}, but not in the follicular phase [76]. Previous studies have found that elevated blood histamine levels during premenstrual syndrome can be alleviated by increasing serum DAO activity with oral magnesium or calcium [77]. This also reminds us of the need to consider the effects of gender, menstrual cycle, and nutrients when assessing serum DAO activity as a means of identifying various diseases.

Alcohol and drugs are also notable inhibitors of DAO. Sessa found that ethanol inhibited DAO activity while acetaldehyde stimulated DAO activity [78,79], and there are as many as 94 known inhibitors of DAO, such as dihydrazine, nafamostat, isoniazid, d-tubocurarine, pancuronium, clavulanic acid, promethazine, verapamil, metoclopramide, numerous antibiotics, etc. [68,80,81]. All of them induce allergic reactions by inhibiting DAO activity. The mechanism of inhibition is sometimes competitive, as with dihydralazine and pancuronium bromide, and sometimes non-competitive (e.g., pentazocine), which may be of particular importance for long-term treatment. It has also been shown that drugs such as Lianshu preparation, berberine, apocynin, and phosphatidylcholine can improve intestinal mucosal barrier damage and protect the integrity of the intestinal mucosa by enhancing the activity of DAO enzymes [82–85].

Histamine-induced food intolerance affects the quality of life of a large proportion of the population, and both DAO deficiency and/or histamine receptor upregulation can exacerbate the symptoms of histamine toxicity. Thus, effective measures must be taken to manage these patients.

4. Low-Histamine Diet/DAO Supplements

To address these clinical diseases and possible mechanisms, a variety of DAO supplements have been developed, and a low-histamine diet is recommended for symptomatic relief and prognostic improvement. Research has shown that treatment with a low-histamine diet only reduces symptoms in HIT patients, and there is no specific drug available to cure [86]. Commercially available dietary supplements, such as Daosin or products from DR Healthcare, are commonly used to provide exogenous porcine DAO to supplement endogenous DAO in the human small intestine through protein derived from porcine kidneys [87]. However, DAO extracted from porcine kidneys does not yield sufficient DAO activity for clinical application at present [66,88]. Dietary treatment of HIT (low-histamine diet and DAO supplementation) can ameliorate intestinal dysbiosis by reducing the relative abundance of histamine-secreting bacteria (i.e., Pseudomonas spp., Aspergillus spp., Aspergillus spp., and Lachnospira spp.) and increasing the bacterial flora associated with intestinal health, thereby improving the clinical symptoms of HIT [89,90].

Interestingly, a recent study has shown that the enzyme-linked UHPLC-FL (ultra-high performance liquid chromatography and fluorimetric) technique has the advantage of rapid, reliable, and highly specific detection of DAO activity in food matrices and could be used as a tool to validate foods with the potential to treat HIT and help in the discovery of more DAO dietary supplements [91].

5. Laboratory Methods for Quantitative Measurement of Serum DAO Concentrations

Given the role of DAO as a diagnostic marker in the identification of gastrointestinal disorders, migraine, pregnancy monitoring, the GI tract toxicity of drugs, hepatitis and post-hepatitis cirrhosis, HIT, and other diseases, a reliable and accurate method for quantification of DAO antigens is necessary.

Strictly speaking, the most direct and reliable method of diagnosing DAO activity should be an assay of intestinal mucosal DAO activity performed on colonic tissue sampled during a colonoscopy. However, intestinal DAO activity is inaccessible in terms of direct measurement and few studies have been conducted on this diagnostic method, with current studies mainly focusing on the methodological measurement of serum DAO activity and values. Previous studies have demonstrated that serum DAO activity is closely related to intestinal nucleic acid and protein synthesis and may reflect the severity of intestinal mucosal damage [92].

DAO activity has been assayed based on the detection of hydrogen peroxide, aldehyde, or dioxygen by spectrophotometric, titrimetric, manometric, fluorometric, polarographic, amperometric, biometric, and radiometric techniques [93–95]. Radioactive methods using a combination of isotope dilution and gas flow counting are the most reliable. However, isotope dilution is a more time-consuming and laborious method, while the liquid scintillation technique is more sensitive, simple, and fast for radioactive determination of DAO activity, making it suitable for large-scale sample measurements [96]; this method is thus referred to as the "gold" standard method for DAO activity determination. Schwelberger et al. prepared five monoclonal antibodies targeting human DAO by immunizing mice with in vitro-expressed fragments of human DAO protein and found that the detection of DAO was 100-fold more sensitive than the most sensitive enzyme assays currently available. Owing to the increased sensitivity of the new monoclonal antibody, DAO expression and cellular localization in various human tissues can be validated, and DAO can also be detected at sites where DAO enzymatic activity has not previously been clearly demonstrated, such as in urine [97].

A new ELISA quantification method described by Boehm et al. allows accurate measurement of DAO concentrations in various biological fluids, showing high agreement with radioactivity assays. Accurate and reliable ELISA assessment of DAO may be used to validate the role of DAO as a potential biomarker in various diseases [98]. Since enzyme activity can be abnormal in patients with normal DAO levels, it is suggested that a link between DAO concentration and activity be made for the clinical diagnosis of various diseases. Beltrán-Ortiz et al. constructed a method which simultaneously determines DAO serum concentrations using the immunodiagnostic DAO ELISA K8500 kit and the standard colorimetric method for determining serum DAO activity, and they found that it was more specific for HIT diagnosis; interestingly, the concurrent application of the two tests was effective in reducing false-positive and false-negative results in HIT patients [59].

However, the reference values for DAO levels in serum have not yet been established, and the measured DAO values and activity in serum do not correspond to those in the intestinal mucosa [99]. In some cases, serum DAO enzyme activity assays can also be considered as a supplementary test if intestinal mucosal DAO activity assays are not feasible.

DAO belongs to the group of copper amine-containing oxidases [100]. As a product of AOC1 gene encoding, DAO preferentially degrades histamine and various polyamines, such as putrescine or spermidine [101]. However, as the main enzyme for the extracellular degradation of histamine, whether DAO exists in the serum or plasma of non-pregnant healthy individuals is controversial. Schwelberger et al. found that purified plasma amine oxidase (PAO) from porcine plasma could efficiently convert histamine and N-methylhistamine, inactivating various amines in the circulation (including histamine); he thus concluded that DAO was not normally present in the bloodstream [102]. This group also failed to detect DAO in human serum through Western blotting in another trial, suggesting that the enzyme activity detected in plasma or serum may be mediated by vascular adhesion protein-1 (VAP-1, also known as PAO) rather than DAO [97]. This differs considerably to conclusions previously reported on the measurement of serum DAO activity, which may be related to the inability of monoclonal antibodies to detect DAO in blood, at least not in relevant quantities.

The main role of DAO is the degradation of extracellular histamine, which has been heavily studied in recent years. DAO catabolizes other polyamines, such as putrescine,

spermidine, and cadaverine, and cadaverine in particular may be a better selective substrate for DAO [103–105]. Meanwhile, other amines may act as competing substrates and interfere with the degradation of histamine by intestinal DAO [106]

6. Conclusions and Future Perspectives

DAO level measurements are useful for the early diagnosis of inflammatory bowel diseases, acute mesenteric ischemia, and other intestinal pathologies. DAO also plays an essential role in a wide range of clinical applications, including detecting various allergic diseases, liver diseases, and pregnancy-related diseases, and can also be used to evaluate the GI toxicity of anticancer drugs. Gender, menstrual cycle, and various foods or drugs that affect the determination of DAO levels pose problems for the reliability of the enzyme in diagnosing clinical disease. At the same time, this can provide novel ideas for the treatment of various diseases, such as HIT and histamine toxicity. Current studies on the use of a low-histamine diet/DAO supplementation mainly focus on HIT and are not widely available. In addition, analysis of blood DAO levels is challenging, and the high economic cost of DAO assays may be the reason why DAO activity assays are not yet widely accepted in daily clinical practice. Routine analysis has been limited to enzymatic assays, which are often less sensitive and impractical, and their specificity and operability still require further optimization.

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Article

Basal Serum Diamine Oxidase Levels as a Biomarker of Histamine Intolerance: A Retrospective Cohort Study

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Abstract: Background: Histamine Intolerance (HIT) is a multifaceted pseudoallergic disorder possibly due to defective histamine metabolism. Diamine oxidase (DAO) contributes to histamine degradation and can be measured in the serum. The role of DAO measurement in the diagnostic work-up of HIT still remains unclear, and conflicting results have been reported in the literature. Therefore, we aimed to evaluate the possible clinical usefulness and consistency of DAO value ranges as provided by the assay manufacturer and verify whether they could predict the response to treatment. Methods: We retrospectively analyzed 192 outpatients with HIT symptoms and measured serum DAO values at baseline. Patients were prescribed either with low-histamine diet and/or enzymatic supplementation according to symptom severity and re-evaluated six to eight months later. Patients were stratified into three groups according to DAO levels: <3 U/mL, 3–10 U/mL, and >10 U/mL. HIT severity was assessed on a scale of 1 to 5 before and after treatment. Results: A total of 146 patients completed the study. Gastrointestinal and cutaneous symptoms, often associated with headache, were more frequent in subjects with DAO < 10 U/mL. Symptom severity and DAO ranges were correlated. Patients with intermediate DAO levels (3–10 U/mL) showed a more complex clinical phenotype but also a more significant improvement in symptom severity (score reduction 50%, interquartile range (IQR) = 33–60%) when compared to patients with low DAO (40%, IQR = 20–60%; $p = 0.045$) or high DAO (33%, IQR = 0–50%; $p < 0.001$). Complex clinical phenotypes were also more frequent in patients with intermediate DAO levels. Conclusions: HIT is characterized by typical symptoms and low levels of DAO activity. Symptom severity was associated with the degree of DAO deficiency. Patients with DAO values between 3 and 10 U/mL show the best response to treatment (low-histamine diet and/or DAO supplementation). DAO value could arguably be considered as a predictor of clinical response to treatment. Prospective studies are needed to confirm these data.

Keywords: diamine oxidase; histamine intolerance; low histamine diet; food intolerance; food supplements

1. Introduction

Food intolerance is defined as an adverse food reaction without the typical pathophysiological and clinical features of overt immune-mediated disorders [1]. It affects more than

20% of the population in industrialized countries [2]. Food intolerance is receiving increasing attention both from research [3,4] and in routine allergy clinical practice possibly due to the many and heterogenous overlapping symptoms with other clinical conditions. The term histamine intolerance (HIT) refers to a complex disorder of controversial definition [4–7], characterized by pseudo-allergic symptoms encompassing post-prandial malaise, diarrhea, and meteorism along with cutaneous manifestations, such as urticaria and itch, as well as vasomotor disturbances leading to hypotension, tachycardia, flushing, rhino-conjunctival symptoms, and headache [8–13]. HIT is supposed to be caused by an imbalance between histamine intake and catabolism [14]. Histamine overload may be due to histamine-rich diets, excessive intake of alcohol, and/or inducers of endogenous histamine release [15–17].

A quantitative and/or functional deficit of diamine oxidase (DAO), the main extracellular enzyme devoted to histamine catabolism along the digestive tract, might also contribute to the pathogenesis of HIT [8–11], while the heterogeneous distribution of histamine receptors might account for clinical phenotype variability among subjects. The diagnostic work-up of HIT still remains challenging, uncertain, and time-consuming as well as frequently leading to misdiagnosis. As a consequence of this, the prevalence of HIT is underestimated, also in light of the high prevalence of potential confounders, such as food allergy, irritable bowel syndrome, lactose intolerance, and non-celiac gluten sensitivity, which may coexist with or constitute alternative diagnoses to HIT [6,12,13,18–20]. At present, the diagnosis of HIT is mostly achieved clinically either by the exclusion of other conditions and by a beneficial response to low-histamine diet [4,21–23] and/or DAO supplementation [24,25]. Oral provocation test with liquid histamine has been proposed by some authors with contradictory results. However, this procedure is not common in clinical practice due to safety concerns and to the absence of standardized doses of histamine to be administered [26–28].

DAO supplementation might improve histamine-related cutaneous symptoms in patients with low basal serum DAO levels [24]. Although some authors suggest the measurement of serum DAO concentration or activity to support HIT diagnosis, there is still no consensus on the optimal use of this biomarker and on the potential reference values useful for diagnosis and/or treatment [8,25,29].

To address this issue, we designed a retrospective study aiming to assess the ability of DAO reference ranges as provided by the radio-extraction assay manufacturer to identify homogeneous groups of HIT patients with distinct degrees of symptoms severity and distinct classes of treatment response to either the low-histamine diet and/or enzymatic supplementation.

2. Materials and Methods

2.1. Patients

Upon informed consent under the Panimmuno research protocol (approved by the Institutional Review Board at San Raffaele Hospital, Milan, Italy, reference code 22/INT/2018), 192 patients with HIT were enrolled in an observational retrospective study among 304 patients with suspected HIT referred to the allergy outpatient clinic of the same institution from 1 January 2018 to 31 January 2020. As per the local practice protocols, patients with suspected HIT underwent a complete allergy work-up, including clinical interview, skin-prick tests, and total and specific Immunoglobulin E (IgE) when needed (Supplementary Materials Table S1). Serum DAO activity was also measured in all patients with suspected HIT through a commercially available radio extraction assay (DAO-REA®; Sciotec Diagnostic Technologies GmbH, Tulln, Austria) used in previous studies [9,30]. All patients with a confirmed new HIT diagnosis were prescribed a low-histamine diet trial and/or DAO supplementation (Daosin®, Sciotec Diagnostic Technologies GmbH, Tulln, Austria: one tablet before each meal or as needed) according to the physician's evaluation of HIT severity (Supplementary Materials Figure S1). The diagnosis of HIT was based on the presence of typical symptoms as described by Tuck et al. [2]. Additional inclusion criteria included age $\geq$ 18 years and availability of at least one serum DAO level measurement

performed within one month before enrollment. Patients with a recent diagnosis of celiac sprue or inflammatory bowel disease, patients who were already on a low-histamine diet or on chronic anti-inflammatory or histamine-releasing drugs, and patients with a recent infection or those who underwent a recent antibiotic therapy were excluded from the study. Furthermore, patients who were not compliant to the treatment were excluded from the analyses. We stratified patients into three groups according to serum DAO levels defined by the assay manufacturer: group 1 DAO < 3 U/mL, group 2 DAO 3–10 U/mL, and group 3 DAO > 10 U/mL.

2.2. Symptom Evaluation

Data collection at enrollment encompassed demographics, comorbidities, HIT symptoms in patient history and at time of enrollment (including postprandial gastrointestinal disturbances, itch and urticaria, dizziness, hypotension, or headache) [29], and concomitant medication. The frequency of HIT symptoms was categorized into three classes: (a) daily; (b) more than 3 times a week; and (c) less than three times a week. HIT symptom grading was adapted from the irritable bowel syndrome severity scoring system [31]. It was recorded at baseline and after a minimum of six and a maximum of eight months. Specifically, symptom severity was graded into 1 = very mild, 2 = mild, 3 = moderate, 4 = severe, and 5 = very severe.

2.3. Statistical Analysis

Due to the low number of subjects and the non-normal distribution of continuous variables, non-parametric tests (Kruskal–Wallis and Wilcoxon tests, as appropriate) were employed to compare continuous variable trends among groups. Fisher's exact test was used to analyze the association between categorical data. *p*-Values below 0.05 were considered significant. Continuous variables are expressed as median (interquartile range, IQR) unless otherwise specified. Categorical variables are reported as absolute numbers (percentage). Microsoft Excel 2019® (Microsoft Corp., Redmond, WA, USA), GraphPad Prism version 9 (GraphPad, San Diego, CA, USA), and the OpenEpi online suite (www.openepi.com, accessed on 25 January 2022) were used for statistical analysis.

3. Results

3.1. Characteristics of the Study Population

Of 192 patients enrolled, 46 were excluded after a few months for incomplete treatment compliance. A total of 146 patients completed the study: 118 women (80.8%) and 28 men (19.1%). The median (IQR) DAO levels at baseline was 8.17 (4.14–15.42) U/mL. There were 31 patients with low (21%, group 1), 60 with intermediate (41%, group 2) and 55 with high (38%, group 3) baseline DAO serum levels. Food allergy was more frequent in group 3 (14/55, 25%) than in group 1 and/or 2 (11/91, 12%, $\chi^2 = 5.774$, $p = 0.033$). DAO groups did not show further differences in terms of demographics or comorbidities (Table 1).

Table 1. General characteristics of the study population.

Features	Group 1 DAO < 3 U/mL *n* = 31	Group 2 DAO 3–10 U/mL *n* = 60	Group 1 + 2 DAO ≤ 10 U/mL *n* = 91	Group 3 DAO > 10 U/mL *n* = 55
Age: median (IQR)	45 (35–57)	43 (35–54)	43 (35–55)	41 (31–54)
Women: *n* (%)	25 (81%)	52 (87%)	77 (85%)	41(75%)
Food allergy: *n* (%)	5 (16%)	6 (10%)	11 (12%) *	14 (25%)
Respiratory allergy: *n* (%)	7 (22%)	25 (41%)	22 (24%)	21 (38%)
Drug allergy: *n* (%)	10 (32%)	21 (35%)	31 (34%)	18 (33%)
Lactase deficiency: *n* (%)	6 (19%)	8 (13%)	14 (15%)	10 (18%)
Total IgE > 100 IU/mL: *n* (%)	5 (16%)	17 (28%)	22 (24%)	17 (31%)
Contact dermatitis: *n* (%)	8 (26%)	21 (36%)	29 (32%)	17 (31%)

IQR, interquartile range; IgE, Immunoglobulin E; DAO, diamine oxidase. * $p < 0.05$ vs. group 3.

3.2. HIT Symptoms before and after Treatment

Patients who presented with a combination of gastrointestinal symptoms, mucocutaneous manifestations, and headache were more frequent in group 2 (20%) than in group 3 (5%, $\chi^2 = 5.353$, $p = 0.038$). The association among gastrointestinal symptoms, mucocutaneous manifestations, headache, and vasomotor symptoms was also more frequent in group 2 (10%) than in group 3 (0%, $\chi^2 = 5.803$, $p = 0.036$). There were no other differences in the prevalence of HIT symptoms among the three DAO groups.

Symptom frequency was significantly higher in group 1 compared to group 2 and group 3. HIT symptom severity was inversely correlated with baseline DAO levels (rho = −0.467; $p < 0.001$). Accordingly, symptom severity was lower in patients with DAO > 10 U/mL compared to patients with intermediate or low DAO levels considered either singularly or together. After treatment with low-histamine diet and/or DAO supplementation, a median reduction of 40% (20–50%) in symptom severity was observed compared to baseline ($p < 0.001$ by signed-rank test). Symptom severity improvement was lower, even if not statistically significant, in group 3 (33%, IQR = 0–50%) than in group 1 (40%, IQR = 20–50%; $p = 0.062$) or group 2 (50%, IQR = 33–60%, $p < 0.001$) or in pooled group 1 + 2 (50%, IQR = 25–60%; $p < 0.001$). Symptom improvement in group 2 was also higher than in group 1 ($p = 0.045$; Table 2). Consistently, symptom severity improvement was inversely correlated with baseline DAO levels (rho = −0.303; $p < 0.001$). Symptom severity scores at baseline were inversely correlated to symptom severity scores after treatment in patients with high baseline DAO levels (rho = −0.438; $p = 0.002$) but not in patients with low or intermediate DAO levels.

Table 2. HIT clinical features.

	Group 1 DAO < 3 U/mL *n* = 31	**Group 2 DAO 3–10 U/mL *n* = 60**	**Group 1 + 2 DAO ≤ 10 U/mL *n* = 91**	**Group 3 DAO > 10 U/mL *n* = 55**
HIT Symptom Prevalence				
Gastrointestinal only	5 (16%)	5 (8%)	10 (11%)	7 (13%)
Skin only	8 (26%)	8 (13%)	16 (18%)	16 (29%)
Headache only	0	0	0	1 (2%)
Gastrointestinal + skin	11 (35%)	23 (38%)	34 (37%)	22 (40%)
Gastrointestinal + headache	1 (3%)	1 (2%)	2 (2%)	3 (5%)
Skin + headache	1 (3%)	5 (8%)	6 (7%)	0
Skin + vasomotor	0	0	0	1 (2%)
Gastrointestinal + skin + Headache	3 (10%)	12 (20%) *	15 (16%)	3 (5%)
Gastrointestinal + skin + vasomotor	0 ^	0 ^	0	2 (4%)
Gastrointestinal + skin + headache + vasomotor	2 (6%)	6 (10%) *	8 (9%)	0
HIT symptom frequency				
Daily	27 (87%) *** ^	36 (60%) **	63 (69%) ***	16 (29%)
> 3 times/week	3 (10%) *** ^	18 (30%) *	21 (23%) **	28 (51%)
< 3 times/week	1 (3%)	6 (10%)	7 (8%)	11 (20%)
HIT symptom severity				
Before treatment	4 (4–5) ***	4 (4–5) ***	4 (4–5) ***	3 (3–4)
After treatment	3 (2–3)	2 (2–2) §§ **	2 (2–3)	2 (2–3)

HIT, Histamine Intolerance; * $p < 0.05$, ** $p < 0.010$, *** $p < 0.001$ vs. group 3, ^ $p < 0.05$ vs. group 2, §§ $p < 0.010$ vs. group 1.

4. Discussion

We retrospectively analyzed a cohort of patients with suspected HIT in order to assess the role of serum DAO measurement in identifying clinically distinct subsets of HIT and differential response to treatment. We found that the majority of these patients had baseline DAO levels below 10 U/mL, in line with most of the literature [10]. Patients with low

(<3 U/mL) or intermediate (3–10 U/mL) DAO levels had a lower prevalence of food allergy compared to patients with higher DAO levels, consistent with a limited role of this comorbidity as a clinical confounder. Symptom severity and frequency were also higher in patients with low or intermediate DAO levels as compared to patients with higher DAO levels. HIT symptom severity was inversely correlated with baseline DAO levels. Accordingly, symptom severity was lower in patients with DAO >10 U/mL compared to patients with intermediate or low DAO levels considered either singularly or together. These data possibly confirm that DAO measurement identifies a pathogenetically relevant mechanism in the development of HIT [8,10,32–34].

Treatment with a low-histamine diet and/or DAO supplementation was generally effective at reducing symptom severity. Patients with higher DAO levels had lower rates of therapeutic success compared to patients with low to intermediate DAO levels. This suggests that additional factors might contribute to HIT symptoms in this category of subjects. Consistently, symptom improvement was proportional to baseline symptom severity in patients with DAO > 10 U/mL but not in the other patient groups, suggesting that DAO activity rather than clinical features at presentation is more relevant in predicting treatment responses in patients with *bona fide* abnormal DAO [24]. Nonetheless, intermediate DAO levels identified a subgroup of patients with more complex, less constant, and more treatment-susceptible symptoms compared to patients with low DAO levels. These data possibly suggest that fluctuating histamine levels due to insufficient but not abolished DAO activity reserve might cause more detrimental clinical manifestations than constitutive histamine overload, which might instead lead to downstream pathway desensitization at least in selected organ/tissues [9,24,35–38]. Conversely, reduced dietary histamine load through diet and oral DAO supplementation might be insufficient to induce symptom remission in patients with complete DAO deficiency.

Taken together, our data support the usefulness of serum DAO measurement as a marker of disease severity and a predictor of treatment response in patients with HIT, consistent with previous evidence in the literature [10,24]. Nonetheless, other groups reported no association among DAO levels and HIT diagnosis, questioning its diagnostic value. At least part of this discrepancy can be explained by the existence of methodological differences in the assays used to measure DAO levels [7,39] and in the gap between in vitro experiments and real-life epistemology [40].

Multiple sets of clinical criteria for HIT diagnosis have been proposed in the literature with the aim of minimizing the confounding effects of other conditions, such as food intolerances [35,41,42], irritable bowel syndrome [43–46], and non-celiac gluten sensitivity [19,47,48]. In the absence of a universal definition of HIT, a reliable comparison among distinct cohorts remains challenging [49]. In addition, there is also limited knowledge about the possible compensatory mechanisms accounting for the coexistence of low DAO levels and the absence of symptoms among healthy subjects as well as on the potential role of pathogenic mechanisms other than DAO deficiency in patients with HIT and higher DAO levels. Regarding this aspect, it might be well-accepted that serum DAO levels alone cannot substitute accurate history taking and conventional allergy and gastroenterological work-up to rule out alternative diagnoses [49]. Indeed, data from this study further highlight that accurate pre-test stratification of patients might enhance the diagnostic significance of DAO measurement, which might be more fit to identify patients with distinct degrees of symptom severity and treatment susceptibility within the spectrum of HIT rather than surrogating other tools for HIT diagnosis [10,24,26].

When interpreting the evidence provided in this study, a set of potential limitations should, however, also be considered. First, our study consisted in a time-limited observation of clinical events occurring in a relatively small although well-characterized cohort of patients with HIT treated with a combination of low-histamine diet and/or DAO supplementation as per routine clinical practice. Larger prospective trials with longer follow-up and homogeneous treatment protocols are required to validate our results and possibly identify more specific subsets of patients for whom the diagnostic and mechanistic role of

DAO could be selectively more relevant. In particular, patients treated with diet should be split from those treated with DAO supplementation. In this context, repeated histamine measurement before and after meals could also be useful to better define the complex picture of HIT in addition to DAO level assessment. Second, as our study was retrospective in nature and focused on "real-life" clinical practice, we did not acquire data from healthy subjects who could have served as a comparator group and might have provided further hints on DAO variability in the general population levels [24]. Third, data from repeated samples were not available. This hampers a thorough evaluation of the possible effects of DAO supplementation on its metabolism and constitutes an additional limitation to the definition of intraindividual DAO variability [10]. Fourth, there is some evidence that does not support the reliability of tests, such as the DAO-REA for the assessment DAO activity. However, other groups have shown that measurement of DAO through the DAO-REA test is able to robustly identify patients with distinct genetic variants of the DAO gene [9], which in turn are known to affect DAO activity. In the study by Maintz et al. [9], DAO levels measured by DAO-REA proved also to be effective at identifying patients with HIT. Finally, the evaluation of symptom severity was based on simplified questionnaires [50], which might cause loss of information on the nature and variability of HIT symptoms in relation with DAO levels and concomitant treatment. Notwithstanding these limitations, the results of our study have the strength of providing comprehensive "real-life" data from a cohort of patients with HIT, possibly enhancing their translational potential to a wide set of subjects commonly seen in allergy and gastroenterology practice.

5. Conclusions

Basal serum DAO levels might contribute to HIT diagnostic work-up by identifying clinically distinct subsets of patients with specific degrees of disease severity and response profiles to treatment. Patients with DAO levels between 3 and 10 U/mL might show the most complex clinical presentation but also the best response performance to treatment with low-histamine diet and/or DAO supplementation. Validation of these results in larger cohorts is needed to translate this explorative evidence into clinical practice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu14071513/s1, Table S1: Positive skin test/PATCH test or specific IgE in the study population. Figure S1: Treatment of patients with possible HIT.

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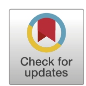

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Standardization of a colorimetric technique for determination of enzymatic activity of diamine oxidase (DAO) and its application in patients with clinical diagnosis of histamine intolerance

Camila Beltrán-Ortiz, Teresa Peralta, Verónica Ramos, Magdalena Durán, Carolina Behrens, Daniella Maureira, Maria A. Guzmán, Carla Bastias and Pablo Ferrer*

ABSTRACT

Background: Diamine Oxidase (DAO) has an essential role for degradation of exogenous histamine in the intestine; thus, histamine intolerance (HI) mainly has been correlated to a low concentration and/or activity of this enzyme. The objective of the study was to standardize a colorimetric technique to measure the enzymatic activity (function) of hDAO to then apply it to a series of 22 patients with a clinical diagnosis of HI.

Methods: For the standardization variables such as volume and type of sample, incubation time, wavelength of maximum absorption, types of substrates, and concentration of oxidized ascorbate were evaluated. Then the activity and concentration of DAO was determined in 22 patients diagnosed with HI and 22 healthy subjects.

Results: The mean of serum DAO concentration in the 22 patients was of 9.268 $\pm$ 1.124 U/mL. The mean of serum DAO concentration in the 22 controls was of 20.710 $\pm$ 2.509 U/mL, being significantly higher (P value 0.0002) the mean of the samples. The mean of serum DAO activity of the patients was of 1.143 $\pm$ 0.085 U/L and the controls was 1.533 $\pm$ 0.119 U/L, significantly greater than the patients (P value 0.011). In addition, the sensitivity of both techniques was 0.63. In the measuring of DAO concentration the specificity was 0.9, constituting a good diagnostic test, especially to rule out the true negatives. The determination of DAO activity had a specificity of 0.68.

Conclusions: Although we used a small number of patients and controls and the absorbance values were lower than expected, statistically significant differences were found in the levels of concentration and DAO activity between the patients with histamine intolerance and the controls. Therefore, the measuring of DAO concentration and DAO activity is a good diagnostic strategy for

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study suspect cases of HI. The simultaneous use of both assays allows to reduce positive and negative false results, for example, patients with normal DAO levels that could present a dysfunction in the activity of this enzyme.

Keywords: DAO, Diamine oxidase, Histamine intolerance, Histamine, Activity

BACKGROUND

Human diamine oxidase (hDAO) is codified by the AOC1 gene, also called ABP1 gene ($\approx$10 kbp), that is located in chromosome 7q35.[1] This homodimeric enzyme of 751 aminoacidic residues has one active site in each subunit; this active site contains a cooper ion and a topaquinone residue formed by the post-translational modification of a tyrosine.[2,3] In each active site hDAO quickly performs oxidative deamination of the polyamine sperimidine, the diamines cadaverine and putrescine, and the monoamines: Histamine and *N*-methilhistamine.[4,5]

hDAO is expressed in various tissues and organs including the brain. However, it is highly expressed in placenta, kidney, and intestinal mucosa.[6,7] hDAO plays an essential role in the intestine being crucial for degradation of exogenous histamine. Histamine intolerance (HI) is a pathology that occurs when there is a dysregulation between an excessive intake of histamine through food, or a deficit in its degradation by the detoxification system at the intestinal and hepatic level. Thus, HI mainly has been correlated to a low amount (concentration) and/or activity (function) of hDAO[1,8,9]

Currently, in Chile our laboratory is the only one that measures serum hDAO concentration as an analysis to be considered when establishing a diagnosis of HI, being a minimally invasive, inexpensive and easily accessible test. Since patients with normal hDAO levels could present a dysfunction in activity of this enzyme, it is recommended to correlate the concentration and hDAO activity with the clinical diagnosis of HI.

The objective of the study was to standardize a colorimetric technique to measure the enzymatic activity (function) of hDAO to then apply it to a series of 22 patients with a clinical diagnosis of HI. For this, variables such as volume and type of sample, incubation time, wavelength of maximum absorption, types of substrates, and concentration of oxidized ascorbate were evaluated.

METHODS

Patients and controls

In this study we employed 22 serum samples from patients (cases) older than 18 years, with a clinical history of HI. The patients were classified as HI according to that described by Rosell-Camps with slight modifications.[9] We used 2 or more histamine-mediated symptoms, with at least 1 gastrointestinal symptom, with negative skin tests in which IgE-mediated food allergy was ruled out, by means of a clinical history associated with skin tests and/or specific IgE. As control we used 22 serum samples from healthy subjects, older than 18 years, without a clinical history of HI, without kinship with the subjects under study, without diseases associated with alterations of the immune system such as cancer and autoimmunity, chronic liver damage, severe burns, kidney damage, and non-pregnant women. In Table 1 we show the epidemiological and clinical characteristics of cases and controls. All study participants signed an informed consent.

Determination of DAO concentration

The concentration of DAO in serum was determinated using the kit DAO ELISA K8500 from Inmunodiagnostik (Germany). Briefly, the

	Cases (n:22)	Controls (n:22)
Female (n)	20	14
Age (years mean)	40,7 (21-62)	35,2 (24-59)
Gastrointestinal Symptoms		
Abdominal Pain (%)	77	0
Diarrhea (%)	45	0
Vomit (%)	13	0
Bloating (%)	63	0
Skin Symptoms		
Flushing (%)	68	0
Urticaria (%)	77	0
Angioedema (%)	27	0
Pruritus (%)	86	0
Respiratory Symptoms		
Nasal Congestion (%)	36	0
Airway obstruction (%)	23	0
Other symptoms		
Headache (%)	72	0

Table 1. Epidemiological and clinical characteristics of patients and control population

kit contains an anti-DAO antibody and a secondary antibody conjugated with streptavidin peroxidase and uses tetramethylbenzidine (TMB) as a substrate. The absorbance of the yellow chromogen produced was determined at 450 nm. The kit used has an analytical sensitivity indicated as limit of detection (LoD) or limit of quantification (LoQ) of 0.120 U/mL. Moreover, include for interpretation of results a reference range: levels <3 U/mL are equivalent to a high probability of HI; levels between 3 and 10 U/mL at a probable HI and levels >10 U/mL at a low probability of HI.

Measurement of the DAO activity

To implement the measurement of the enzymatic activity of DAO, the technique published by Takagi et al (1994)[10] was standardized using serum or plasma. To achieve the optimal conditions for the measurement of enzymatic activity different volumes of sample, concentrations of substrates, and reagents were used. To find the wavelength of greater absorption, readings were made at different wavelengths. Briefly, this technique is based on the measurement of DAO's ability to degrade cadaverine, a process in which hydrogen peroxide is generated. Then chromogen DA-67 was used, which is quantitatively oxidized in the presence of hydrogen peroxide and peroxidase, to be transformed into methylene blue (Fig. 1). To eliminate the interference caused by the presence of ascorbic acid in the serum, an interfering agent of the peroxidase, we used the enzyme ascorbate oxidase. Finally the reaction was stopped with sodium diethyl-dithiocarbamate (stop solution), allowing the formation of methylene blue, which remained stable for 2 h, measured the absorbance with a maximum absorbance of 668 nm. Incubations at 37 °C were performed in a thermo regulated bath.

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DAO

$H_2N(CH_2)_5NH_2 + O_2 + H_2O \rightarrow H_2N(CH_2)_4CHO + H_2O_2 + NH_3$

Cadaverine — 5-Amino pentanal

POD

$DA\text{-}67^{*} + H_2O_2 + H_3{}^{+}O \rightarrow \text{Methylene blue}^{**} + 2H_2O + HOOCNHCH_2COONa$

(λ max=668nm) — 2-Amino(N-carboxy) sodium acetate

* 10-(Carboxymethylaminocarbonyl) -3, 7-bis(dimethylamino) phenothiazine sodium salt

** 3, 7-Bis (dimethylamino) phenothiazine-5-ium chloride

Fig. 1 The principle of the assay method of diamine oxidase (DAO) activity. The amount of the methylene blue formed as an enzymatic oxidative product was spectrophotometrically measured at 668 nm (Figure obtained from article published by Takagi et al. 1994, reference N°10)

Statistical analysis

For the statistical analysis between the averages of the different determinations, the unpaired student t-test was used, considering a confidence interval of 95%. In addition, to analyze the discriminative capacity of the techniques used to determine the amount and activity of DAO, we used the ROC (Receiver Operating Characteristic) curve.

RESULTS

Volume optimal of sample

To implement the determination of DAO activity in serum, the technique published by Takagi and collaborators in 1994 was used as a starting point. For the standardization, the volume of sample needed was first analyzed (Fig. 2). For this test a DAO standard of 25 U/L (Fig. 2A, B and 2C) and serum from a control subject (Fig. 2D and E) were employed. In Fig. 2A, the absorbance obtained was compared when using 100 and 200 μL as sample. Mean ± SD absorbance using 100 μL was of 0.4530 ± 0.0008, and using 200 μL was of 0.8120 ± 0.0016, being significantly higher ($P < 0.0001$). In Fig. 2B, the absorbance was compared when using 200 and 300 μL. The absorbance obtained using 200 μL was of 1.016 ± 0.0205, and using 300 μL was of 1.395 ± 0.0144, being significantly higher ($P < 0.0001$). For its part, comparison between 100, 200, and 300 μL of serum of a control subject in D showed that absorbance is not detectable when using 100 μL of serum; absorbance obtained using 200 μL was of 0.0107 ± 0.0079, and using 300 μL was of 0.0377 ± 0.0061, being significantly higher ($P = 0.0189$). Finally, comparisons between 200 μL and 400 μL of DAO standard of 25 U/L and serum of a control subject are shown in Fig. 2C and E, respectively. The absorbance obtained by using 200 μL of standard was of 0.7160 ± 0.0114 and with 400 μL was of 1.236 ± 0.0347, being significantly higher ($P < 0.0001$). The absorbance obtained by using 200 μL of serum of a control subject was

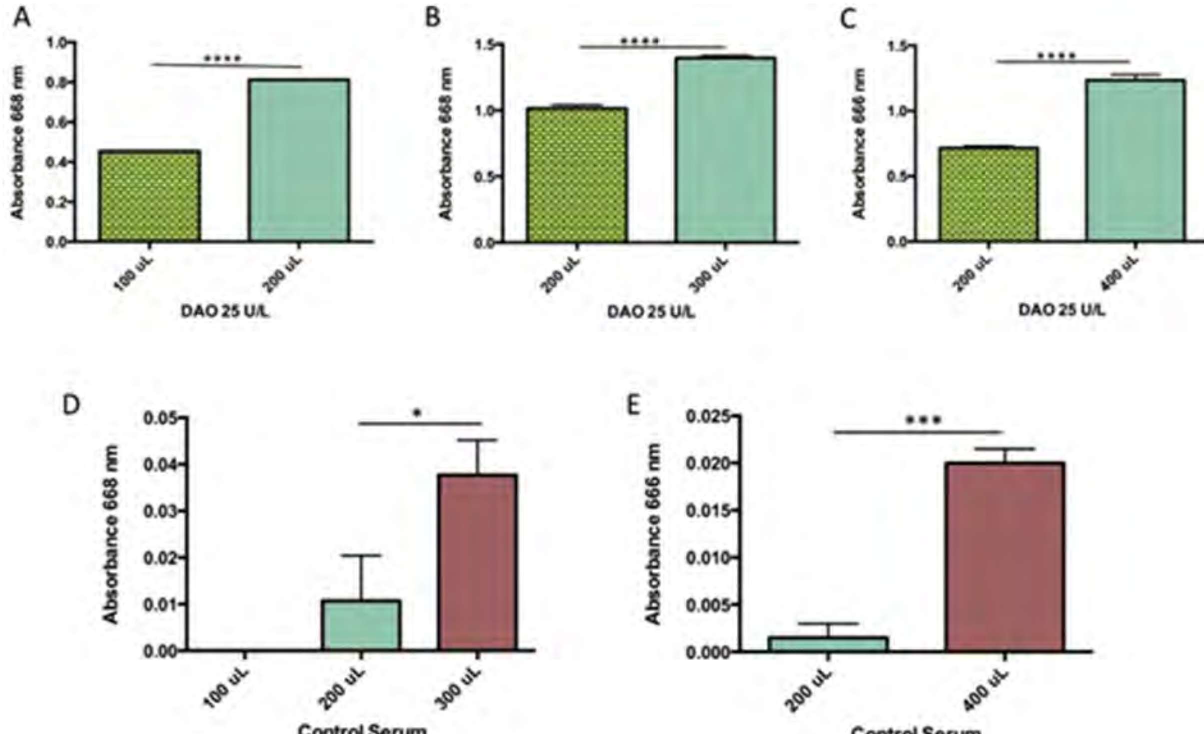

Fig. 2 Standardization of volume of sample for determination of DAO activity. The technique described by Takagaki et al. was used varying the volume of sample A) Comparison between 100 and 200 μL of DAO 25 U/L. B) Comparison between 200 μL and 300 μL of DAO 25 U/L. C) Comparison between 200 μL and 400 μL of DAO 25 U/L. D) Comparison between 100 μL, 200 μL and 300 μL of serum of a control subject. E) Comparison between 200 μL and 400 μL of serum of the same control subject. In all the determinations there was significant difference ($P < 0.05$), obtaining the highest absorbance when using 400 μL of standard DAO 25 U/L or serum of a control subject

of 0.0015 ± 0.0012 and with 400 μL was of 0.0200 ± 0.0012, being significantly higher ($P = 0.0001$). These results show that the optimum sample quantity is 400 μL, maximum volume allowed by the physical conditions in which the technique was performed. Unlike Fig. 2A,B and D, the absorbance assays plotted in Fig. 2C and E were measured at 666 nm.

Determination of optimal wavelength

Although in the article published by Takagaki[10] a fixed wavelength of 668 nm was used to measure the absorbance of methylene blue, we considered that the absorbance values obtained for them were too much low. For this reason we scanned between 666 and 6672 nm to determine if there was another wavelength that gave absorbance values higher than obtained by Tekagi. We tested with various samples and determined that in spite of not having a significant difference, in all the assays the highest absorbance was recorded at 666 nm (data not shown).

Time for incubation of the samples

The incubation times for formation of hydrogen peroxide and methylene blue described by Takagaki et al[10] were also analyzed. However, increasing the incubation time increased the signal of the samples and the target; therefore, there was no real increase in absorbance (data not shown).

Analysis of substrates for determination of DAO activity

In addition, in the standardization we compared the results obtained when determining the activity of DAO in serum using the substrates cadaverine, histamine, and putrescine (Fig. 3). In Fig. 3A and B, 30 mM cadaverine, 30 mM Histamine, and 60 mM Histamine were compared using 300 μL of DAO standar 25U/L or serum control, respectively. With DAO 25 U/L (Fig. 3A) Mean ± SD absorbance of 1.3953 ± 0.0144 was obtained using 30 mM Cadaverine, 0.3987 ± 0.0046 with 30 mM Histamine and 0.2670 ± 0.0168 with

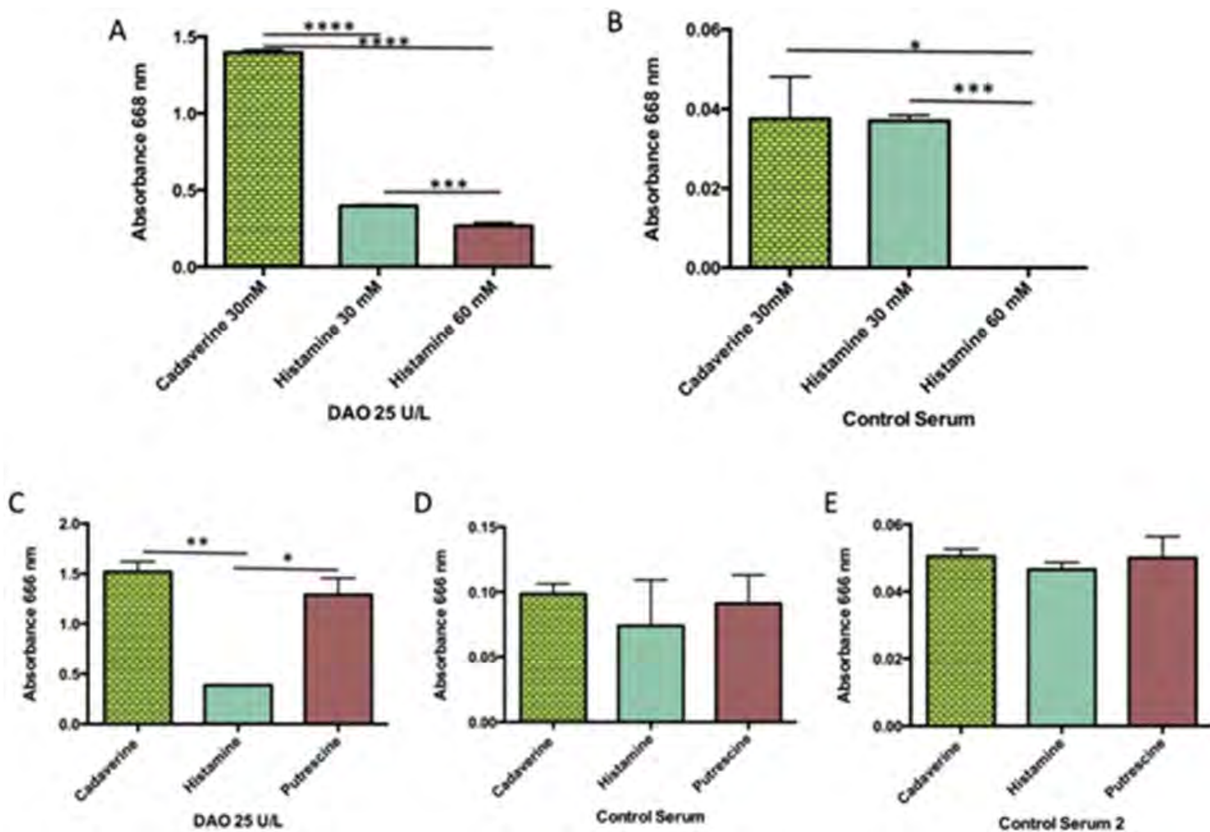

Fig. 3 Analysis of different substrates for determination of DAO activity. In this analysis the technique described by Takagaki et al. was used, varying the substrate with 300 μL of DAO standard 25 U/L o control serum in A y B, respectively. In C, D and E 400 μL of sample and 30 mM concentration of each substrate were used. In A and B we compared cadaverine with histamine, given that histamine is a monoamine we used the twice concentration to compare. With 25 U/L DAO standard cadaverina proved to be significantly better substrate that histamine (P value $<$ 0.0001). In B, using a serum control no significant difference between cadaverine and histamines was shown. In C, Cadaverine and Putrescine showed to be significantly better substrates than histamine, but in D and E using different serums controls no significant difference was shown between the substrates

60 mM histamine. Cadaverine demonstrated a significantly higher absorbance than 30 or 60 mM histamine ($P < 0.0001$). In addition, the absorbance of 30 mM histamine was significantly greater than 60 mM histamine ($P = 0.0004$).

Using serum of a control subject (3B), an absorbance of 0,0375 $\pm$ 0,0075 was obtained using 30 mM cadaverine, 0.0370 $\pm$ 0.001 with 30 mM histamine, and when 60 mM histamine was used, no signal was detected. Although 30 mM histamine and 30 mM cadaverine are significantly better than 60 mM histamine ($P = 0.0007$ and $P = 0.0377$ respectively), there is no significant difference between them.

In Fig. 3C-E, 30 mM concentration of cadaverine, histamine, and putrescine were used. In C, 400 μL of DAO standard 25 U/L were used; cadaverine 1.519 $\pm$ 0.071 and putrescine 1.291 $\pm$ 0.116 showed a significantly higher absorbance than histamine 0.3855 $\pm$ 0.0015 (P value 0.0039 and 0.0161, respectively). In Fig. 3D and E, 400 μL of serum of different control subjects were used; in both there was no significant difference between the different substrates. Due to these results, we maintained cadaverine as the substrate.

Determination of optimal concentration of ASOD

Given that previous results (Fig. 2) established a 4-fold increase in the volume of sample used for the determination of DAO activity in serum, we analyzed the increase the volume of 30 mM cadaverine solutions in PIPES buffer and color solution, described by Takagaki et al. Both proved to be in sufficient quantity and even decreased the absorbance when we increased the volume (data not shown).

Also, the amount of ascorbate oxidase (ASOD) needed was determined (Figure supplementary 1). Results obtained when using 5 or 20 U/mL of ASOD were compared. On the left is the comparison using 400 μL of standard DAO 25 U/L. In this assay, the absorbance obtained using 5 U/mL of ASOD was 1.236 ± 0.0425, and with 20 U/mL of ASOD was 1.225 ± 0.021. To the right, the comparison is shown using 400 μL of serum from a control subject; in this case the absorbance obtained using 5 U/mL of ASOD was 0.020 ± 0.002, and with 20 U/mL of ASOD was 0.033 ± 0.005. Both with the DAO 25 U/L standard and the serum of the control subject, there was no significant difference when comparing ASOD 5 U/mL and 20 U/mL ($P = 0.8376$ and $P = 0.2116$, respectively). Although using the serum of a control subject the absorbance using 20 U/L of ASOD is greater, it is not significant and numerically negligible. For this, we demonstrated that 5 U/mL of ASOD is enough.

Choosing plasma or serum for DAO activity assay

Finally, a comparison between serum and plasma (of heparinized tubes) of control subject was made for determination of enzymatic activity of diamine oxidase (Fig. 4). All the samples correspond to the same control subject. In addition, 5 mL of serum and plasma were ultracentrifuged and 1 mL from the bottom of the tube of each was collected and used to determine the utility of ultracentrifugation. Mean ± SD absorbance obtained using serum was of 0.06125 ± 0.00225, with plasma was 0.0625 ± 0.0020, using ultracentrifuged serum was 0.06125 ± 0.00825, and with ultracentrifuged plasma was 0.0635 ± 0.0130. There was no significant difference between serum, plasma, and both ultracentrifuged.

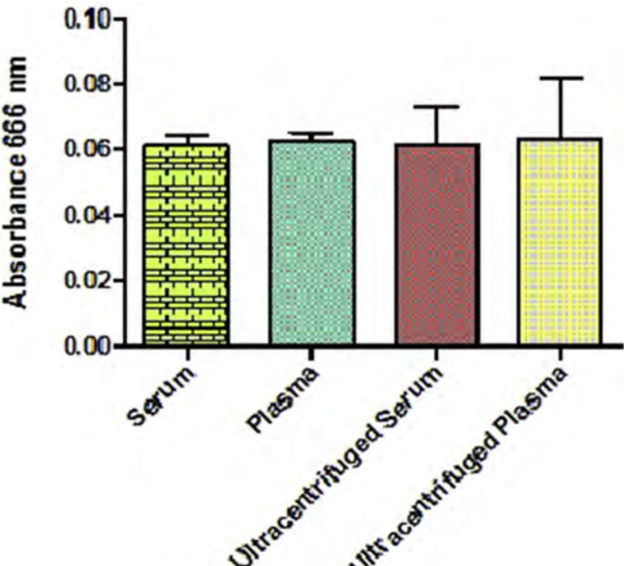

Fig. 4 Comparison between serum and plasma of control subject for determination of enzymatic activity of diamine oxidase. In this analysis, the technique described by Takagaki et al[10] was performed, using 400 μL of sample, and setting absorbance measurement to 666 nm. Serum and plasma (of heparinized tubes) of the same control subject was compared. In addition, 5 mL of serum and plasma were ultracentrifuged, and the lower volume of each was collected and used to determine the utility of ultracentrifugation. There was no significant difference between serum, plasma, and ultracentrifuges.

Concentration and DAO activity in patients and controls

With these results, the optimal conditions for the implementation of the DAO activity serum determination were standardized (Fig. 5). Briefly, 1.5 mL of substrate solution (25 mM PIPES buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100) must be incubated at 37 °C for 5 min. After adding 400 μL of the serum sample, it should be incubated at 37 °C for 30 min. Then, 1.5 mL of color solution (100 μM DA-67, 6 U/mL peroxidase and 5 U/mL ascorbate oxidase in 25 mM MES buffer (pH 5.4), containing 0.5% Triton X-100) must be added and incubated at 37 °C by 1 h. Finally, 50 μL of the stop solution (30 mM sodium diethyldithiocarbamate solution) is added, mixed, and the absorption must be measured at 666 nm against the blank solution. Porcine kidney DAO solution (5, 10, 15, 20 and 25 U/L, in 25 mM buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100, are used as standard.

As reference range was established, levels <0.6 U/L are equivalent to a high probability of HI; levels between 0.6 and 1.3 U/L at a probable HI and levels >1.3 U/L at a low probability of HI.

Having standardized the technique, we proceeded to determine the concentration and DAO activity in patients and controls (Fig. 6). The mean of serum DAO concentration in the 22 patients was of 9.268 ± 1.124 U/mL. While, the mean of serum DAO concentration in the 22 controls was of 20.710 ± 2.509 U/mL being significantly higher (P value 0.0002) the mean of the samples. The mean of serum DAO activity of the patients

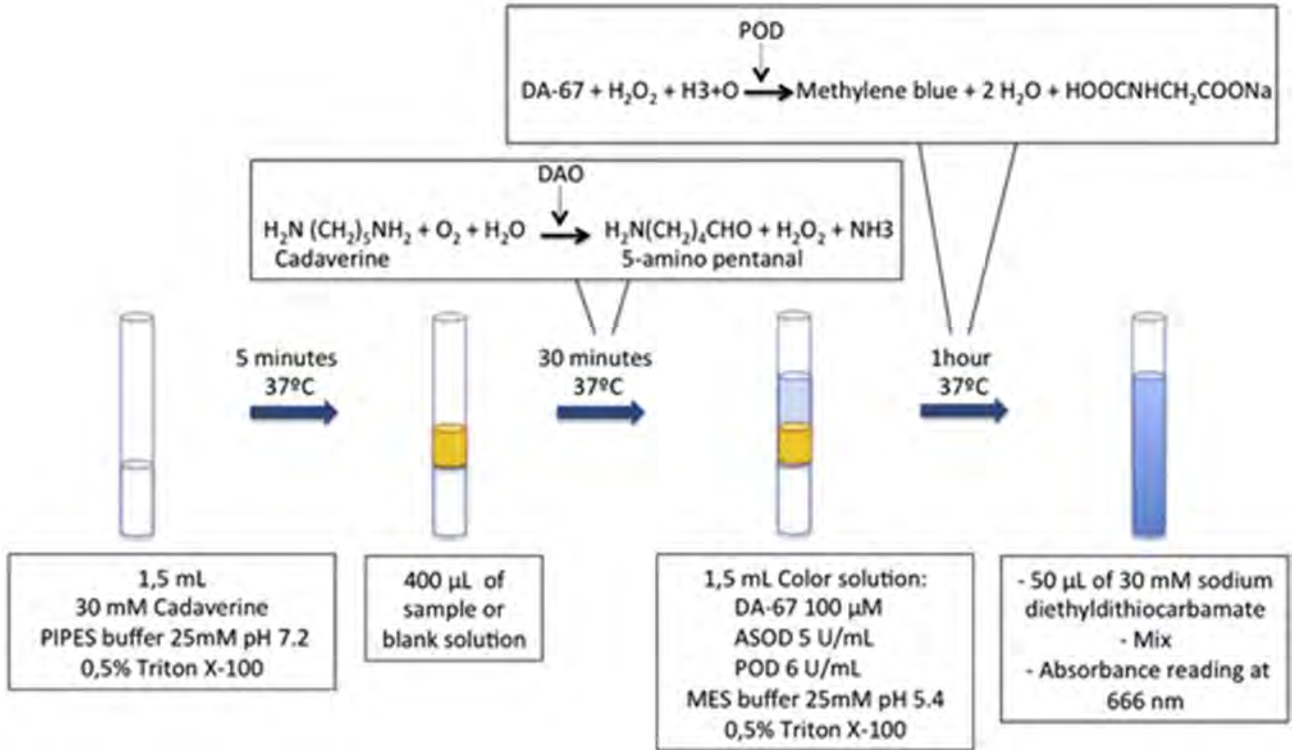

Fig. 5 Summary of the optimal conditions for the determination of DAO activity. 1.5 mL of substrate solution (25 mM PIPES buffer (pH 7.2), containing 30 mM cadaverine and 0.5% Triton X-100) must be incubated at 37 °C for 5 min. After adding 400 µL of the serum sample (or blank solution), it was incubated at 37 °C for 30 min. Then, 1.5 mL of color solution (100 µM DA-67, 6 U/mL peroxidase and 5 U/mL ascorbate oxidase in 25 mM MES buffer (pH 5.4), containing 0.5% Triton X-100) was added and incubated at 37 °C by 1 h. Finally, 50 µL of 30 mM sodium diethyldithiocarbamate solution was added, mixed, and the absorption was measured at 666 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was of 1.143 ± 0.085 U/L, and the controls was 1.533 ± 0.119 U/L, significantly greater than the patients (P value 0.011).

Additionally, we compared the average of concentration and DAO activity between female and male patients and control as indicators of Chilean people. The mean DAO concentration in men was of 22.760 ± 2.668 U/mL. The mean of DAO concentration in women was of 19.540 ± 3.674 U/mL. On the other hand, the mean of DAO activity of the men was of 1.388 ± 0.162 U/L, and the mean of DAO activity of the women was of 1.616 ± 0.162 U/L. In both comparisons no significant difference was observed.

Finally, we constructed the ROC curve for the techniques used for the determination of the concentration and DAO activity (Fig. 7). The sensitivity of the technique employed to measure the serum DAO concentration was 0.63 and the specificity 0.9. For this part, the standardized colorimetric technique for determination of

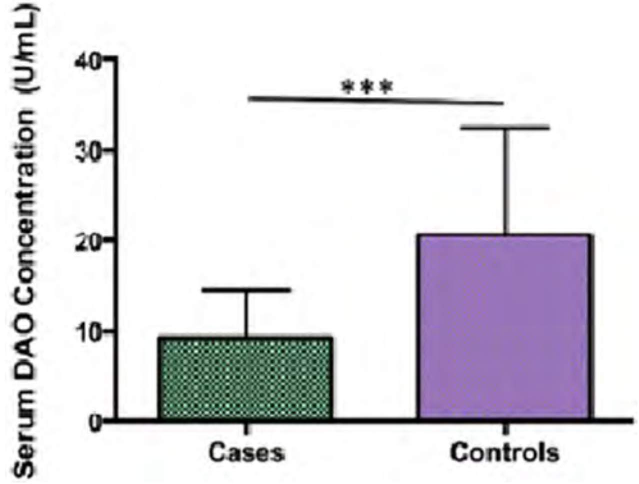

Fig. 6 Determination of concentration (left) and DAO Activity (right) in patients (cases) and controls. DAO activity was determinated using the technique standardized. The mean of DAO concentration in the 22 patients was of 9.268 ± 1.124 U/mL. While, the mean of DAO concentration in the 22 controls was of 20.710 ± 2.509 U/mL being significantly higher (P value 0.0002) the mean of the samples control. DAO activity was assayed using the technique described by Takagaki et al., using 400 µL of sample and setting absorbance measurement to 666 nm. The mean of DAO activity of the patients was of 1.143 ± 0.085 U/L and the controls 1.533 ± 0.119 U/L was significantly greater than the cases (P value 0.0109)

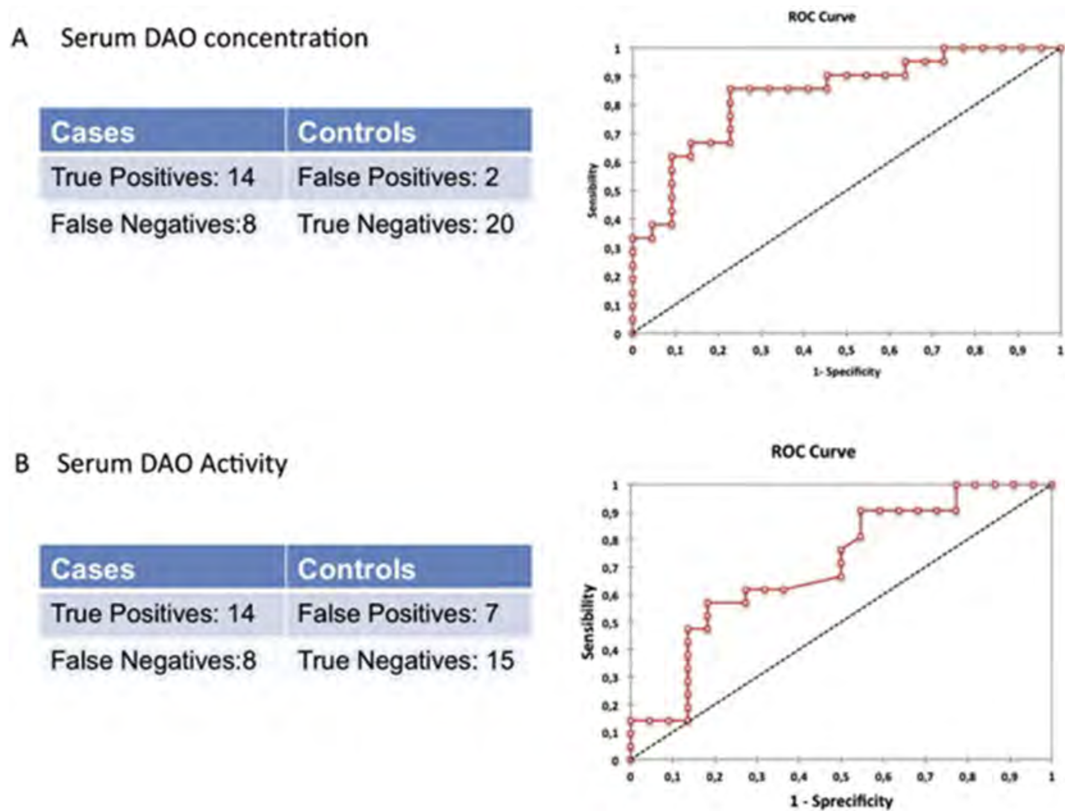

Fig. 7 ROC curve for concentration and DAO activity. The sensitivity of the technique employed for DAO quantification was 63% and the specificity 90%; constituting a good diagnostic test for the determination of DAO concentration. On the other hands, the standardized colorimetric technique used for the determination of DAO activity showed a sensitivity of 63% and a specificity of 68%. Therefore, the DAO activity assays is adequate for reduce false negatives results obtained for the DAO concentration analysis

Serum DAO activity has a sensitivity of 0.63 and specificity of 0.68. It is important to mention that although the specificity is lower, the serum DAO activity (functional) analysis established that 3 of the 8 false negatives established for the determination of the DAO concentration (37.5%) had probability of HI.

DISCUSSION

Since patients with normal hDAO levels could present a dysfunction in activity of this enzyme, it is recommended to correlate the concentration and hDAO activity with the clinical diagnosis of HI. To implement the determination of DAO activity in serum of Chilean patients classified as intolerant to histamine, the technique published by Takagi et al in 1994[10] was standardized. Although the conditions described in the original technique presented good values when using a DAO standard, these do not necessarily represent physiological conditions, this is reflected in that the signal was very low when using serum samples (in both, cases and controls). For this, the first standardization analysis that we established was of 400 µL as the optimum sample quantity (Fig. 2), maximum volume allowed by the physical conditions in which the technique was performed. Given the 4-fold increase in sample volume, it was shown that the amount used of reagents and incubation times are sufficient.

Also, a sweep test was carried out to various samples determining that in spite of not having a significant difference, in all the tests the highest absorbance was recorded at 666 nm (data not shown), thus, we established the absorbance at 666 nm.

The technique described by Takagi et al employs cadaverine as a substrate;[10] however, DAO has been shown to have different affinity for the various amines on which it acts.[1,2,4,5]

In fact, the main interest of the technique is to establish the activity of DAO with respect to the oxidative deamination of histamine. Given this, in the standardization we compared the results obtained when determining the activity of DAO in serum using the substrates cadaverine, histamine, and putrescine (Fig. 3).

The use of serum samples did not show a significant difference between 30 mM cadaverine, 30 mM histamine, and 30 mM putrescine; this is probably due to the low absorbance obtained by these samples (Fig. 3B,D and E). Several analyzes were carried out to exclude the presence of interfering in the serum that would justify this low absorbance (data not shown), the results obtained rule out the presence of interferences and indicate that the low absorbance is probably related to a low DAO activity not only in people with HI but also in the general population.

By using the DAO 25 U/L standard, different absorbance to different substrates was shown, which is related to the variable activity described between them. Also, a significantly better absorbance with cadaverine and putrescine with respect to histamine (Fig. 3A and C) was shown, although kinetic studies reported in the biography describe a preference greater of DAO to histamine than to putrescine or cadaverine.[1,5] This could be due to low stability of the histamine, which when we analyzed at double the concentration, considering having an equal quantity of available amines, resulted in a significantly lower absorbance when using DAO standard 25 U/L (Fig. 3A), and signal was not obtained when using control serum (Fig. 3B). For these results, although it would have been ideal to determine the activity using histamine as a substrate, we decided that the cadaverine was maintained.

Finally, the use of serum or plasma (heparinized tubes) from the same control subject with and without ultracentrifugation was compared to determine the DAO activity (Fig. 4); there was no significant difference between serum, plasma, and ultracentrifuged. As a result of this analysis, we can induce that, given the impossibility of working with serum, the plasma derived from heparinized tubes could be used for the determination of DAO activity as in other techniques,[11] although we prioritized to maintain the sample originally described. Ultracentrifugation did not prove useful. Once we had the standardized technique (Fig. 5), we proceeded to determine the serum DAO activity and serum DAO concentration of controls and cases of HI (Fig. 6). In both, the average of controls was significantly higher than in the cases with a diagnosis of HI. Additionally, this is the first study that allowed characterization of DAO concentration and DAO activity in a Chilean population, being 20.71 $\pm$ 2.509 U/mL and 1.533 $\pm$ 0.1188 U/L, respectively. In the literature the occurrence of gender-related variability in DAO activity in healthy individuals has been previously reported.[12] For these cases, the average of serum DAO concentration and serum DAO activity was compared between men and women using the control as an indicator of Chilean people. In both comparisons no significant difference was observed.

Finally, the ROC curve for the techniques used for the determination of the serum concentration of DAO and the DAO serum activity were constructed (Fig. 7). The sensitivity of both techniques was 0.63. In the technique used to measure the concentration of DAO in serum, the specificity was 0.9, which constitutes a good diagnostic test, especially to rule out the true negatives. For other part, the standardized colorimetric technique for determination of serum DAO activity had a specificity of 0.68. Although the specificity is lower, the serum DAO activity (functional) analysis established that approximately one third of false negatives established by measuring the concentration of DAO in serum had probability of histamine intolerance, being a good complementary method in order to reduce false negatives, since patients with normal DAO levels could present a dysfunction in the activity of this enzyme for example due to single nucleotide polymorphisms.[13]

By way of projection, we expect to evaluate these techniques by comparing the functional DAO data obtained by using a commercial KIT that uses Histamine as a substrate (D-HIT, Sciotec, Austria), which has been used to correlate DAO activity in serum as a diagnostic test of HI.[14]

Also, an uncomplicated pregnancy is believed to be dependent on the balance between DAO and histamine in the placenta,[15] and low serum DAO Activity has been correlated to gastrointestinal toxicity and malnutrition due to anticancer drugs.[16] For this reason, it would be worth evaluating the relevance of these techniques for monitoring the functioning of DAO in pregnancy and in oncologic treatments.

In conclusion, our results indicate that a colorimetric technique to measure the activity (function) of DAO present in human serum or plasma could be standardized. Although we used a small number of patients and controls and the absorbance values were lower than expected, statistically significant differences were found in the levels of concentration and DAO activity between the patients with histamine intolerance and the controls. Therefore, the measuring of DAO concentration and DAO activity is a good diagnostic strategy for study suspect cases of HI. The simultaneous use of both assays allows to reduce positive and negatives false results, for example, patients with normal DAO levels that could present a dysfunction in the activity of this enzyme.

Also, in Chilean people, serum DAO concentration and serum DAO activity in men and women do not present a significant difference.

Abbreviations

DAO: Diamine Oxidase; HI: Histamine Intolerance; ELISA: Enzyme-Linked Immuno Sorbent Assay; ROC: Receiver Operating Characteristic; hDAO: Human Diamine Oxidase; TMB: Tetramethylbenzidine; ASOD: Ascorbate Oxidase

Consent for publication

All authors approved the publication of this work.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author, Dr. Pablo Ferrer Campos (E-mail:pferrer40@gmail.com).

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Author's contributions

Camila Beltrán-Ortiz, Biochemist, carried out the experiments of this work and revised the manuscript.
Dr Teresa Peralta designed the project, recruited the patients, and revised the manuscript.
Verónica Ramos, Biochemist, carried out the experiments of this work and revised the manuscript.
Magdalena Durán, Medical Technologist, carried out the experiments of this work and revised the manuscript.
Carolina Behrens, Biochemist, carried out the experiments of this work and revised the manuscript.
Daniella Maureira, Medical Technologist, carried out the experiments of this work and revised the manuscript.
Dr Maria A Guzmán designed the project, recruited the patients, and revised the manuscript.
Dr Carla Bastias designed the project, recruited the patients, and revised the manuscript.
Pablo Ferrer, Biochemist and Dr, PhD, designed the experiments, reviewed all the results, and carried out the final critical review of the manuscript.

Ethics approval and consent to participate

Prior to the study all the patients signed an informed consent approved for the Ethical Committee of the Hospital Clinico Universidad de Chile.

Declarations of competing interest

The authors declare that they have no competing interests.

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Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2020.100457.

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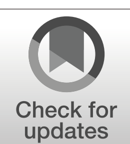

In vitro determination of diamine oxidase activity in food matrices by an enzymatic assay coupled to UHPLC-FL

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Abstract

Intestinal diamine oxidase (DAO) acts as a protective barrier against exogenous histamine. A deficit of DAO activity can lead to the appearance of histamine intolerance, a clinical condition that may be treated by a low-histamine diet and oral DAO supplementation to enhance intestinal histamine degradation. As sources of DAO, porcine kidneys and certain legume seedlings are suitable components for the formulation of a DAO supplement. The aim of this work was to develop a rapid and reliable methodology for the in vitro determination of DAO activity in food matrices based on an enzymatic assay coupled to UHPLC-FL. The proposed method showed a satisfactory linearity and sensitivity and provided a relative standard deviation lower than 3%, guaranteeing method precision, and a mean recovery greater than 99% both for lyophilized pea sprouts and porcine kidney protein extracts. A high specificity is a key attribute of this method due to the use of histamine as the reaction substrate and the direct quantification of its degradation. Moreover, the lack of interference of catalase and hydrogen peroxide is another advantage in comparison with previously published methods. Lyophilized pea sprouts showed the greatest histamine-degrading activity (0.40 ± 0.01 mU/mg), followed by porcine kidney protein extracts (0.23 ± 0.01 mU/mg) and commercial DAO supplements (0.09 ± 0.06 mU/mg). This technique could be used as a tool to validate the DAO activity of food matrices of potential interest for the treatment of histamine intolerance.

Keywords UHPLC-FL · Enzymatic assay · Histamine · Diamine oxidase (DAO) enzyme · Porcine kidney · Pea sprouts

Introduction

The enzyme with histamine-degrading capacity, discovered in 1929 by Charles H. Best in autolyzing lung tissues, was first known as histaminase [1]. After subsequent studies revealed its ability to deaminate other diamines, such as putrescine and cadaverine, the enzyme was renamed diamine oxidase (DAO) [2, 3]. DAO (EC 1.4.3.22), which belongs to the category of copper-containing amine oxidases, is a homodimeric and ubiquitous enzyme found in microorganisms, plants, and animals, generally in the range of 140 to 200 kDa [4–8]. In particular, DAO catalyzes the oxidative deamination of the primary amino group of histamine to imidazole acetaldehyde, consuming dioxygen with the concomitant release of stoichiometric amounts of ammonia and hydrogen peroxide (Fig. 1) [9, 10].

In humans, DAO is mainly located in the intestines, placenta, and kidneys [6, 11]. Intestinal DAO acts as a protective barrier against exogenous histamine, especially of food origin [12–14]. A deficiency of DAO enzyme may thus lead to excess the normal plasmatic levels of histamine (0.3–1.0 ng/mL) and the subsequent appearance of histamine intolerance symptoms [15, 16]. Due to the diverse effects and functions of histamine in multiple organs and systems of the body, histamine intolerance is characterized by a variety of complaints, including gastrointestinal (abdominal pain, diarrhea, or vomiting), dermatological (urticaria, dermatitis, or pruritus), respiratory (rhinitis, nasal congestion, or asthma),

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Fig. 1 Oxidative deamination of histamine by DAO

cardiovascular (hypotonia or arrhythmias), or neurological (headaches) [14–17]. The most frequently used treatment for histamine intolerance consists of following a low-histamine diet [15, 18]. Only foods with histamine levels below detectable limits can be considered safe for histamine-intolerant patients, and unfortunately for this population, histamine is widespread among all food categories in highly variable concentrations [19, 20]. In this context, considering that DAO is the key enzyme in the breakdown of dietary histamine at the intestinal level, orally administered DAO supplements have been proposed as a strategy to enhance histamine degradation and improve the quality of life of intolerant individuals undergoing those dietary restrictions [21, 22]. As sources of DAO, porcine kidneys and certain legume seedlings are suitable components of such an enzymatic supplement [21, 23].

A wide range of methods to detect in vitro DAO activity are described in the literature. With the aim of measuring the rate of substrate degradation or the generation of by-products of this enzymatic reaction, most methods are based on the detection of hydrogen peroxide, aldehyde, or dioxygen by spectrophotometric [13, 21, 24, 25], fluorometric [26], polarographic [27, 28], or amperometric [29, 30] techniques. Radioimmunoassay techniques have also been extensively described, consisting of the radioactive labeling of the substrate and the scintillation counting of its consumption [3, 11, 31]. Although chromatographic analytical procedures are widely used, this approach has only been applied to measure histamine or other biogenic amine degradation capacity in microbial starter cultures involved in food-fermenting processes [10, 32, 33]. Despite some of these methods may be advantageous in terms of rapidity or automation, they generally have a limited sensitivity, require a laborious experimental setup, or entail a high cost in the correct storage and handling of radioactive waste. Moreover, in those methods in which the DAO activity is estimated through the determination of hydrogen peroxide or dioxygen, the action of other enzymes, such as catalase, may interfere by H_2O_2 consuming or O_2 releasing [34, 35]. Additionally, the most extensively used reaction substrates in the methods reported so far are putrescine and cadaverine, which have different affinity or kinetic parameters to histamine [36, 37].

Therefore, the aim of this work was to develop a reliable, rapid, and highly sensitive methodology for the determination of in vitro DAO activity of several matrices using histamine as the substrate and based on the direct quantification of its degradation during the reaction process. Specifically, an enzymatic assay coupled to an ultra-high performance liquid chromatography and fluorimetric (UHPLC-FL) detection method was proposed, validated, and tested for applicability in porcine kidney protein extracts, legume sprouts, and commercialized DAO supplements.

Material and methods

Reagents and chemicals

Histamine dihydrochloride, purified DAO from porcine kidney, and catalase from bovine liver were purchased from Sigma-Aldrich (St. Louis, MO, USA). UHPLC-grade methanol and acetonitrile, hydrochloric acid 0.1M, perchloric acid 70%, sodium di-hydrogen phosphate anhydrous, and di-sodium hydrogen phosphate anhydrous were obtained from PanReac Química (Castellar del Vallès, Spain). Acetic acid, boric acid, 1-octanesulfonic acid sodium salt, ammonium formate, phthaldialdehyde (OPA), and brij® L23 solution were acquired from Sigma-Aldrich (St. Louis, MO, USA); and formic acid, sodium acetate anhydrous, potassium hydroxide, and 2-mercaptoethanol from Merck (Darmstadt, Germany). A LaboStar System from Evoqua Water Technologies (Warrendale, PA, USA) was used to produce ultrapure water (18.2 MΩcm).

Samples

For the analytical method development and validation, porcine kidney protein extracts and lyophilized pea sprouts (*Pisum sativum*) were used. Porcine kidney extracts were provided by a biotechnology company specialized in the extraction of biomolecules from animal tissues. These extracts consisted of a homogenate powder obtained by an acetonic extraction followed by a drying process. Porcine kidney extracts consisted of 84% of protein, estimated by applying the nitrogen-to-protein conversion factor (6.25) to the total nitrogen determined following the Kjeldahl method (2200 Kjeltec® Auto Distillation Unit, Foss Iberia S.A.U., Barcelona, Spain). Etiolated pea sprouts were obtained in our laboratory through the germination of peas at 27 °C and 70% HR. After the sprouts were freeze-dried (Cryodos-50, Telstar, Terrassa, Spain), a lyophilized extract consisting of 39% of protein was obtained. Samples were kept under refrigeration (4–8 °C) until analysis.

The applicability of the method was assayed with 13 different production batches of porcine kidney protein extract, 7

batches of lyophilized pea sprouts and 6 commercialized DAO supplements available in the market. These dietary supplements were in the form of gastro-resistant-coated capsules or tablets, all of them containing 4.2 mg of porcine kidney protein extract.

In vitro determination of DAO activity

The capacity of the DAO enzyme to degrade histamine in a working solution with a defined initial concentration of histamine was tested under controlled optimal conditions (37 °C, pH 7.2). The subsequent analysis of degraded histamine during the reaction time was performed by UHPLC-FL. Specific DAO activity is expressed in mU/mg, referring to the amount of histamine that is degraded by a milligram of sample per minute (nmol of degraded histamine per minute/mg of sample).

Enzymatic assay

Figure 2 illustrates in a schematic manner the experimental procedure of the enzymatic assay for the in vitro determination of DAO activity. In detail, 1 to 20 mg of porcine kidney protein extract, lyophilized pea sprouts, or the content of one tablet or capsule of dietary supplement were thoroughly homogenized in 20 mL of 0.05M phosphate buffer solution (pH 7.2) and placed in a shaker incubator (Ivymen® 100-D, JP SELECTA S.A., Abrera, Spain) for at least 30 min (37 °C, 200 rpm). The addition of a histamine standard solution to reach an initial concentration of 45 µM in the homogenized sample marked the start of the enzymatic reaction. The enzyme in contact with its substrate was kept in constant incubation and 500 µL aliquots were progressively extracted at different sampling times (t = 0, 1, 2, 4, and 6 h). To stop the enzymatic reaction, 15 µL of 2N perchloric acid solution was added to the extracted aliquot, vigorously mixed with a vortex mixer, and centrifuged (4 °C, 5 min, 15000 rpm). The supernatant was filtered through a 0.22-µm GHP filter and stored at 4 °C until UHPLC analysis. Each sample was analyzed in duplicate and a positive control was performed with 1 mg of purified DAO.

To assay the potential interference effect of catalase on the DAO activity determination, porcine kidney protein extract was assayed with the addition of catalase enzyme at two different concentrations (100 and 500 U/mL) using the same experimental procedure.

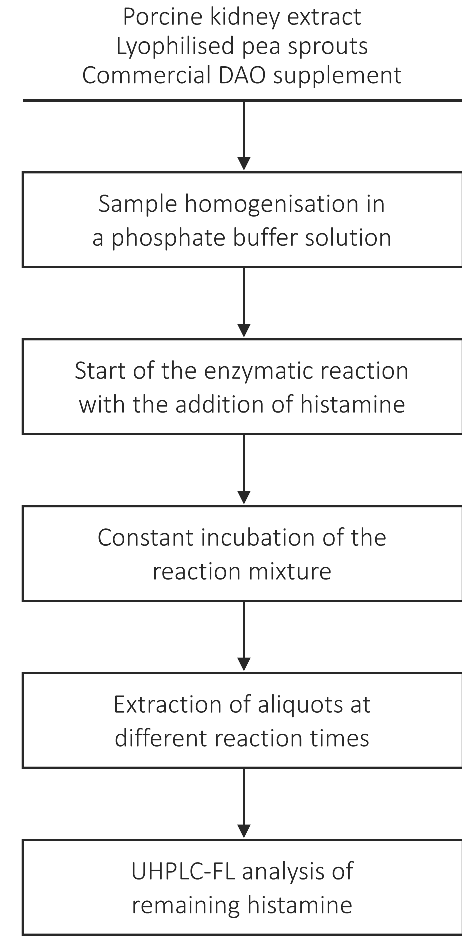


Fig. 2 Schematic experimental procedure of the enzymatic assay for the in vitro determination of DAO activity

UHPLC-FL analysis

Chromatographic separation was performed using a UHPLC-FL system consisting of a Waters Acquity™ Ultra Performance Liquid Chromatography apparatus, which comprised a quaternary pump, an auto-sampler and a fluorescence detector, and a post-column reagent manager connected to a zero dead volume union between the column outlet and the detector. Data acquisition was performed using the Empower™ 3 software (Waters Corp., Milford, MA, USA).

The chromatographic determination of histamine was performed by ion-pair reverse-phase UHPLC coupled with post-column online derivatization with OPA and fluorescence detection. Elution time was 7 min. Chromatographic conditions were as previously described by Latorre-Moratalla et al. [38], briefly summarized in Table 1.

Statistical analysis

The software package IBM SPSS Statistics (IBM Corporation, Armonk, NY, USA) for Windows (version 22) was used for the statistical analysis of data. The reliability of the method was tested by means of analysis of variance for linear regression and the data sets were compared using the Student's t test. Cochran's C test was used to assess the homogeneity of variances.

Table 1 Chromatographic conditions for the UHPLC-FL determination of histamine

Stationary phase	
Column	Acquity UPLC™ BEH C18 column (1.7 μm, 2.1 mm × 50 mm)
Column temperature	42 °C
Mobile phase	
Eluent A	H_2O solution with 0.1 M sodium acetate and 10 mM sodium octanesulfonate (adjusted to pH 4.8 with acetic acid)
Eluent B	H_2O solution with 0.2 M sodium acetate and 10 mM sodium octanesulfonate (adjusted to pH 4.5 with acetic acid): Acetonitrile (6.6:3.4)
Linear gradient	0 min, 80% A; 2 min, 80% A; 3 min, 60% A; 4 min 50% A; 5 min, 40% A; 6 min, 20% A; 6.40 min, 80% A; 7 min, 80% A
Flow rate	0.8 mL/min
Injection volume	1 μL
Fluorescence detection	
Derivatization reagent	OPA (0.2 mg/mL), brij®, 2-mercaptoethanol, methanol, potassium hydroxide and boric acid
Excitation and emission wavelengths	340 nm and 445 nm
Flow rate	0.4 mL/min

Results and discussion

The method developed in this work is based on the direct addition of a defined amount of histamine to a food matrix homogenized in an aqueous solution. During the incubation period of the mixture, the DAO enzyme potentially present in the sample progressively degrades the substrate. DAO activity was determined by comparing the absolute amount of histamine degraded during the reaction time with the initial substrate concentration. The absence of histamine degradation when assaying the same amount of substrate but lacking the DAO enzyme or samples as a negative control proved that the degradation of histamine in the proposed method is exclusively mediated by the enzyme.

The UHPLC-FL method allowed us to unequivocally determine the remaining histamine in the samples with a chromatographic elution time of 7 min and without the need for tedious pre-column derivatization procedures. The selected substrate concentration was 45 μM of histamine, in accordance with published kinetic data for DAO activity on this specific amine, to ensure optimal performance of the enzymatic reaction [9, 39, 40]. The degradation of histamine was monitored for 48 h to study the enzymatic reaction. A linear histamine degradation rate was observed in the first 6 h of the assay ($r > 0.9990$) for both porcine and legume matrices.

Method reliability

The linearity of the method was assessed by performing in triplicate seven determinations of different enzymatic activities using purified DAO and verified by analysis of the variance of the regression. A correlation coefficient of $r = 0.9998$ and a coefficient of determination (r^2) higher than 99% were obtained ($p < 0.001$), demonstrating the satisfactory performance of the method within the DAO activity range of 0.7 to 4.5 mU. Regarding method sensitivity, the limit of detection (LOD) and the limit of quantification (LOQ) were estimated using a regression curve with low DAO activity values and considering the mean response of a blank plus three or ten times the standard deviation of the blank, respectively [41]. Specifically, the value obtained for LOD was 0.025 mU, and for the LOQ, it was 0.038 mU.

The precision and recovery of the proposed method for routine analysis of DAO activity were assessed with different batches of porcine kidney protein extract and lyophilized pea sprouts. The precision was evaluated by performing 7 independent determinations of DAO activity for each food matrix (Table 2). The relative standard deviation was in both cases lower than 3%, showing a satisfactory level of precision. The Horwitz equation for intra-laboratory studies confirmed the acceptability of this precision data [42]. Recovery was evaluated by performing 7 independent determinations of porcine kidney extract and lyophilized pea sprouts, considering 3 addition levels with purified DAO (Table 2). The recovery values obtained for the three levels of addition were satisfactory and no statistical differences from the theoretical value 100% were found ($p > 0.05$) [42]. The variance of the recovery values was not dependent on the content of the analyte according to Cochran's C test ($p > 0.05$).

Among the range of methodologies described in the literature that challenge the determination of DAO activity, the majority are based on the measurement of the liberation of hydrogen peroxide or the consumption of oxygen occurring along the oxidative deamination reaction [13, 21, 24–30]. Those largely used approaches face an important drawback, as the presence of hydrogen peroxide and dioxygen may be

Table 2 Precision and recovery results for porcine kidney extracts and lyophilized pea sprouts

	Precision		Recovery[c]			
	RSD (%)[a]	RSDH (%)[b]	Addition level I	Addition level II	Addition level III	Cochran's test C_{exp}[d]
Porcine kidney extract	2.76	3.45-4.60	100.54 (4.98)	102.69 (5.44)	99.14 (2.52)	0.41
Lyophilized pea sprouts	2.80	3.27-4.36	101.28 (0.90)	100.00 (0.76)	100.51 (2.61)	0.05

[a] Relative standard deviation (RSD) for seven determinations

[b] Acceptable range for relative standard deviations according to the Horwitz equation for intra-laboratory studies (1/2–2/3 of the interlaboratory study calculate by the formula)

[c] Mean recovery percentages and standard deviation in parentheses for three addition levels corresponding to enzymatic activities of 0.5, 1.0, and 2.0 mU for porcine kidney extract and 1.0, 2.0, and 4.0 mU for lyophilized pea sprouts

[d] Cochran's C variance outlier test, C_{tab} (6, 2, 0.05) = 0.8534.

markedly influenced by the concomitant presence of other enzymatic capacities in certain complex biological matrices [34, 35]. This is the case of catalase, an enzyme commonly found in plant and animal tissues, which can lead to the underestimation of DAO activity by consuming H_2O_2 and releasing O_2 [34, 35]. Therefore, the frequent occurrence of catalase in DAO-positive matrices makes those techniques unadvisable due to major interference effect. In this sense, Ahmadifar et al. [35] have recently proposed a zymographic approach consisting in an electrophoretic separation of DAO enzyme followed by its densiometric image analysis capable to evaluate the DAO activity of a sample in the presence of interfering catalase. Concurrently, all those methods consisting in the monitoring of hydrogen peroxide release through a coupled reaction with peroxidase entail further complexities, such as a potential partial substrate inhibition produced by excess of hydrogen peroxide [43]. In general, coupled peroxidase assays may be targeted as unreliable when working with purified DAO enzyme and totally unadvisable when studying non-purified complex samples due to the presence of peroxidase inhibitors or other enzymatic activities [34, 44]. In fact, Calinescu et al. [34] evaluated the DAO capacity of formulation containing a vegetal extract with the presence of catalase, using both a peroxidase-coupled assay and an alternative assay non-related to peroxidase enzyme. In this context, the authors described the unsuitability of the peroxidase-coupled assay due to the diminution of released H_2O_2 by catalase enzyme, emphasizing the need to seek for enzymatic tests not affected by the presence of catalase [34]. In this sense, methods based in the direct measurement of the degradation of the amine substrate, hitherto scarcely described in the literature, may overcome this limitation. In the proposed method, DAO activity of the porcine kidney extract did not significantly differ ($p > 0.05$) when it was determined with or without the addition of catalase, and independently of the concentration of this enzyme added to the sample. Therefore, the proposed method herein is not influenced by the presence of catalase present in the analyzed food matrices, since it is based in the direct determination of histamine.

Although the largely used spectrophotometric techniques seem to be sensitive enough for the analysis of samples with an elevated degree of purification, there is a lack of a reliable and sensitive methods that allow to determine DAO activity in complex biological or food matrices, which will not only contain several potential interferences but will also show relatively low enzymatic rate. In this case, radiochemical detection techniques based on the use of C^{14}-labeled putrescine becomes the preferred approach [44]. However, while a high sensitivity may be attributed to the latter, serious concerns related to the hazardous potential in the handling of radioactive material and the high cost and unsuitability of its storage need to be considered. The proposed method shows the advantages of sensitivity, reproducibility, and automatization of an UHPLC approach while avoiding user-related hazardous potential and becomes a suitable approach to analyze DAO activity in complex non-purified matrices.

Suitability of the method for the determination of DAO activity in porcine kidney protein extracts, lyophilized pea sprouts, and DAO supplements

The applicability of the developed method was tested by analyzing several production batches of porcine kidney protein extract and lyophilized pea sprouts. Additionally, the enzymatic capacity of porcine kidney extract in DAO supplements available in the market was studied. All analyzed products showed in vitro histamine-degrading capacity (Fig. 3). Lyophilized pea sprouts were the most effective, with a mean enzymatic activity of 0.40 (± 0.01) mU/mg, compared with 0.23 (± 0.01) mU/mg for porcine kidney protein extracts. It is worth highlighting that the DAO activity of both products showed minimal variation among different production batches. These results are in good agreement with previously published data indicating a higher catalytic turnover rate for plant- than animal-derived DAO [5, 23, 37]. The amine-degrading capacity described in the literature for these food matrices is highly variable, with values ranging from 0.1 to 500 mU/mg. Different behavior toward the same amino

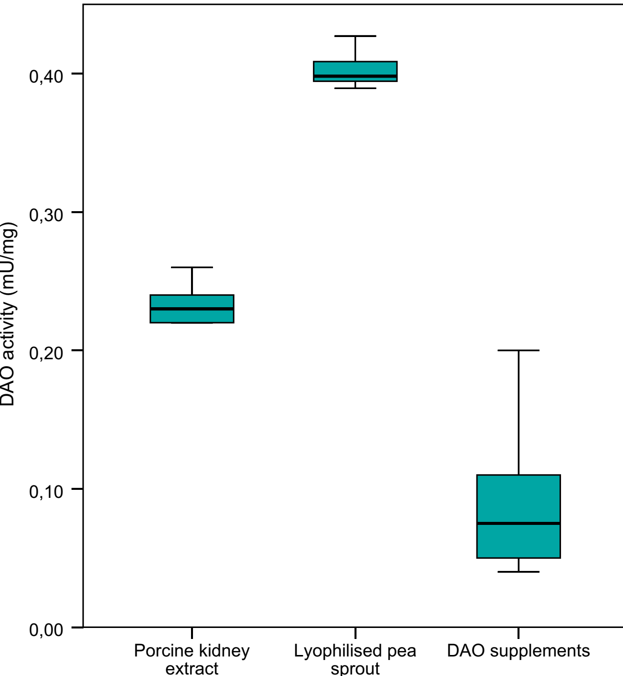
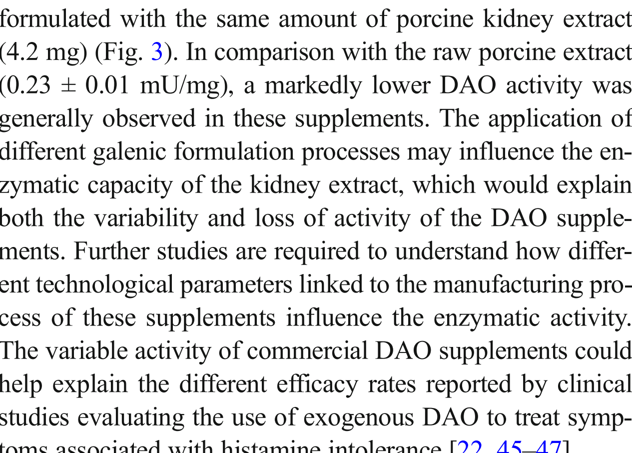


Fig. 3 In vitro DAO activity of several production batches of porcine kidney protein extract, lyophilized pea sprouts, and different commercial DAO supplements

substrates has been reported for DAO enzymes depending on whether they are of animal or plant origin [37]. This heterogeneity could also be explained by differences in methodology between studies, as a range of detection techniques and substrates have been used. Thus, Kivirand et al. [7] suggested that the substrate specificity data available for DAO varied according to the experimental method and recognized an important difficulty to find comparable data due to the evidenced dispersion of methodological procedures. Concretely, the wide range of used substrates (i.e., putrescine, cadaverine, agmatine, histamine, spermidine, and spermine) may easily lead to differences in the reported enzymatic activities, as the affinity of DAO for each substrate varies [36, 37]. Due to the evidenced differences in kinetic parameters depending on the amino substrates, histamine is the optimal substrate in order to have an available methodology to determine the enzymatic activity of potential new sources of DAO, considering the degradation of this target substrate and no other amines.

Porcine kidneys and pea seedlings are the main sources of DAO according to the literature [21, 23], but it can also be found in other food products, such as certain legumes (*Cicer arietinum*, *Lathyrus sativus*, *Lens esculenta*), barley (*Hordeum vulgare*), maize (*Zea mays*), and tea (*Thea sinensis*) [21, 23, 36]. The method proposed here could be applied to validate the in vitro enzymatic capacity of these food matrices and to screen for new potential sources of DAO.

The DAO activity of the six commercial DAO supplements ranged widely from 0.04 to 0.20 mU/mg, despite all being



formulated with the same amount of porcine kidney extract (4.2 mg) (Fig. 3). In comparison with the raw porcine extract (0.23 ± 0.01 mU/mg), a markedly lower DAO activity was generally observed in these supplements. The application of different galenic formulation processes may influence the enzymatic capacity of the kidney extract, which would explain both the variability and loss of activity of the DAO supplements. Further studies are required to understand how different technological parameters linked to the manufacturing process of these supplements influence the enzymatic activity. The variable activity of commercial DAO supplements could help explain the different efficacy rates reported by clinical studies evaluating the use of exogenous DAO to treat symptoms associated with histamine intolerance [22, 45–47].

Few studies have estimated the intestinal DAO activity in a healthy population. An enzymatic activity of 0.001–0.03 mU/mg has been reported in the intestinal mucosa, with higher values given for intestinal protein (0.2–0.33 mU/mg) [31, 48–50]. As indicated by the manufacturers, the usual posology of DAO supplements is 1 capsule before each meal, which provides an enzymatic activity in the range of 0.17 to 0.84 mU, depending on the product. In view of these results, more accurate studies are needed in order to establish the effective dosage of DAO that can provide a complementary intestinal protective barrier for histamine-intolerant individuals.

Conclusion

The proposed method, consisting of an enzymatic assay coupled to a UHPLC-FL technique, allowed the in vitro determination of DAO activity in food matrices using histamine as the reaction substrate. This method provided satisfactory experimental performance in terms of linearity, sensitivity, precision, and recovery, and its suitability was tested on different food matrices reported as sources of DAO. The DAO activity of lyophilized pea seedlings was nearly two-fold higher than that of porcine kidney protein extracts. The histamine-degrading capacity of the six DAO supplements available in the market was variable and lower compared with the other analyzed matrices. Due to the growing awareness of histamine intolerance, it is important to have effective methods for validating the DAO activity of supplements and foods of potential interest for the treatment of this disorder.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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