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Assessment of the resistance of porcine diamine oxidase enzyme to temperature and pH

Relatore: Prof.ssa Sara Spilimbergo
Correlatori: Prof. Juan Carcel Carrion
Prof. José Javier Benedito Fort

Laureanda: ALESSANDRA MINEO

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A mia sorella

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Abstract

The diamine oxidase (DAO) enzyme is naturally produced by humans and allows histamine decomposition. However, when a deficiency of this enzyme exists intolerance or intoxication can occur. The goal of the work was to evaluate the conditions at which the DAO enzyme could be introduced in the processes of preparation of foods rich in histamine, as an alternative to the pharmacological approach. For that purpose, the enzymatic activity of the porcine diamine oxidase (DAO) was studied after treatments at different conditions of pH and temperature.

The resistance to pH was analyzed by exposing the enzyme to pH 3, 5, 7, 9 buffer solutions for 1 hour, 7 days and 60 days at 4°C. The resistance analysis to storage temperature conditions was carried out by keeping the enzyme samples in a neutral buffer solution (pH 7.2) at -18°C and at room temperature (23±1°C) for 1 hour and 7 days. The resistance analysis at temperature conditions of thermal treatments was carried out by keeping the enzyme samples in a neutral buffer solution (pH 7.2) at 40°C, 60°C, 70°C and 80°C for 2 (only for 80°C), 5, 10, 15 and 20 minutes. The enzyme activity of untreated samples was also studied, in order to make comparisons between samples before and after any treatment. The experiments were conducted in triplicate, in each case. The average enzyme activity in terms of U (μmol of degraded histamine/min) and the residual enzyme activity (%) were estimated by a colorimetric method for each treatment. Statistical analysis were carried out in order to find significant differences between treatments and interactions between the factors.

Results showed that the porcine DAO enzyme is only active in neutral or basic media and in these conditions its activity increases during time, specifically up to 60 days of storage at 4°C. Considering storage conditions in neutral buffer: the enzyme maintains its original activity if stored either at -18°C or at room temperature (23±1°C) up to one week of time. Finally, after thermal treatments in neutral buffer at 70°C and 80°C, the DAO activity decreases significantly. Whereas the DAO activity is enhanced at 40°C after 20 minutes of incubation.

Consequent industrial practices should be set to guarantee the desired DAO preservation.

Riassunto

L'enzima diamina ossidasi (DAO) è un prodotto dell'organismo umano e permette la decomposizione dell'istamina. Quando la presenza di questo enzima è sbilanciata rispetto alla quantità di istamina negli alimenti, possono verificarsi fenomeni di intolleranze o intossicazioni. L'obiettivo del lavoro è stato quello di valutare le condizioni in cui l'enzima DAO potrebbe essere introdotto nei processi di preparazione di alimenti ricchi di istamina, come alternativa all'approccio farmacologico. A tal fine, è stata studiata l'attività enzimatica della diammina ossidasi suina (DAO) dopo trattamenti a diverse condizioni di pH e temperatura.

La resistenza al pH è stata analizzata esponendo l'enzima a soluzioni tampone a pH 3, 5, 7 e 9 per 1 ora, 7 giorni e 60 giorni a 4°C. L'analisi della resistenza alle condizioni di conservazione è stata effettuata mantenendo i campioni di enzima in una soluzione tampone neutra (pH 7.2) a -18°C e a emperature ambiente (23±1°C) per 1 ora e 7 giorni. Infine, l'analisi della resistenza ai trattamenti termici è stata effettuata mantenendo I campioni di enzima in una soluzione tampone neutra (pH 7.2) a 40°C, 60°C, 70°C e 80°C per 2 (solo per 80°C), 5, 10, 15 e 20 minuti. È stata studiata anche l'attività enzimatica dei campioni non trattati, al fine di effettuare confronti tra i campioni prima e dopo qualsiasi trattamento. Gli esperimenti sono stati ripetuti tre volte, per ogni trattamento. Attraverso un'analisi colorimetrica sono state stimate l'attività enzimatica media in termini di U (µmol di istamina degradata/min) e l'attività enzimatica residua (%), per ogni campione. Sono state inoltre condotte analisi statistiche al fine di trovare differenze significative tra le condizioni di trattamento ed eventuali interazioni tra i fattori.

I risultati hanno mostrato che l'enzima DAO suino è attivo solo in ambienti neutri o basici e che in queste condizioni la sua attività aumenta nel tempo, in particolare fino a 2 mesi di conservazione a 4°C. Considerando le condizioni di conservazione in tampone neutro: l'enzima mantiene la sua attività originale se conservato a -18°C o a temperatura ambiente (23±1°C) fino a una settimana di tempo. Infine, dopo trattamenti termici in tampone neutro a 70°C e 80°C, l'attività della DAO diminuisce significativamente, mentre questa aumenta se il trattamento avviene a 40°C per 20 minuti.

Conseguenti pratiche industriali dovrebbero essere impostate in modo da garantire la conservazione desiderata dell'enzima.

Chapter 1

Introduction

This section includes introductory information about the topics of histamine, DAO enzyme and histamine intolerance and intoxication phenomena. Finally the approaches to the cure and prevention of histamine intolerance due to DAO deficiency are listed and described, from the oldest to the most recent studies.

1.1 Histamine in foods

Histamine (2- [4-imidazolyl] ethylamine) is a biogenic amine. The name originates from the Greek word *histios* – tissue, which indicates the presence of this substance in many tissues, especially in the lungs, skin, nasal mucosa and stomach [1]. Due to its chemical structure and number of functional groups, histamine can be defined as a heterocyclic diamine consisting of an imidazole ring and one ethylamine functional group [2].

Histamine can be originated naturally inside the human body, where it has got a specific function, or can be introduced from external sources.

This substance derives from the decarboxylation reaction of histidine by the histidine decarboxylase enzyme with the participation of pyridoxal phosphate, the active form of vitamin B6 [3]. The reaction mainly occurs in mast cells, basophils, platelets, histaminergic neurons, and enterochromaffin cells (EC) of the gastrointestinal tract [1]. In the human organism histamine is a neuro-immuno-endocrine system mediator influencing the whole spectrum of physiologic functions of various tissues and cells, including immunity [4].

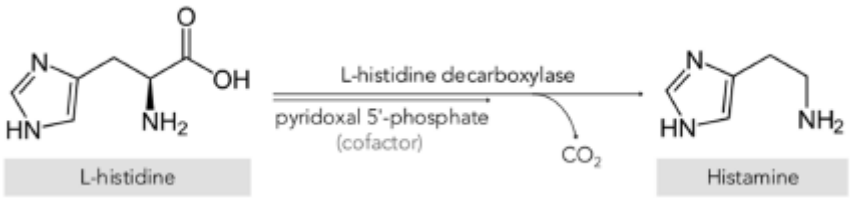

Figure 1-1 *Histamine formation reaction* [5]

Particularly histamine is responsible of processes like the stimulation of gastric acid secretion, inflammation, smooth muscle cell contraction, vasodilation and cytokine production [5]. 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 [6]. The quantity of endogenous histamine is controlled on a genetic level [4], and it is also influenced by psychological stress: the hormones released during stress reaction directly activate mast cells, leading to the release of histamine and other inflammation factors [7].

Histamine is introduced from the outside because of foods rich in histamines and bacteria capable of carrying out the histidine decarboxylation reaction – representatives of the genus *Lactobacillus*, *Pediococcus*, *Photobacterium* or *Klebsiella* [1], so with decarboxylase activity on aminoacids [8]. About foods, there are ones naturally rich in histamine, as fresh products (e.g. tomatoes, spinach, meat, eggs, fish, citrus fruits and dairy products [1]), and others which accumulate it, in high quantities. Usually processing in unsuitably hygienic conditions, fermentation [5], and storage of foods provoke histamine formation and accumulation caused by microorganisms, particularly where the content of L-histidine is high [4]. The explanation of this phenomenon is that the decarboxylation reaction is recognized to be a survival strategy for these microorganisms in acidic environments, as well as an alternative source of metabolic energy in situations of suboptimal substrate availability [9]. Several Gram-positive and Gram-negative bacteria responsible for microbial spoilage or fermentative processes in food are able to produce histamine [5]. Specifically in fish, the Enterobacteriaceae species Hafnai aluei, Morganella morganii and Klebsiella pneumonia have been identified [5]. Whereas in cheeses, fermented meat, vegetable derivatives and fermented beverages, various lactic acid bacteria correspond to histamine-producing microorganisms (e.g., Lactobacillus hilgardii, Lactobacillus buchnerii, Lactobacillus curvatus and Oenococcus oeni), as well as certain strains of Enterobacteriaceae [2].

In order to prevent histamine contamination of food, cooling (4°C) is insufficient since some of these bacteria are able to decarboxylate histidine at these temperatures, so freezing is necessary [7]. While rising the temperature does not produce any desired effect since histamine is a thermostable compund, for this almost irremovable [7]. Moreover even after bacterial autolysis the enzymatic activity of histidine decarboxylase can last, producing more histamine [7].

The concentration of amines formed in foods depends on the type of microorganisms present, the action of decarboxylase enzymes produced by microorganisms on specific amino acids and favourable conditions for enzymatic activity, such as intrinsic and extrinsic parameters like temperature and pH, oxygentension, availability of carbon sources, presence of vitamins, coenzymes and fermentable carbohydrates [8]. Based on the mean content of histamine the food safety of the considered food products can be ranked in the following decreasing order: “dried anchovies”, “fish sauce”, “fermented vegetables”, “cheese”, ”other fish and fish products” and “fermented sausages” [2].

1.2 The diamine oxidase enzyme

Histamine can be metabolized through oxidative deamination carried by diamine oxidase (DAO, also called histaminase)] [3], a functional enzyme acting like a catalyst [5] found in animal tissue and plasma, plants, yeasts, fungi and bacteria [10]. Histamine can also be metabolized by methylation of imidazole core to N4-methylhistamine, catalysed by enzyme histamine N-methyltransferase (HNMT) [7].

DAO is a secretory protein [5] stored in vesicular structures that bonds to plasma membrane of cells, it is released in circulation after stimulation and it is responsible for degradation of extracellular histamine, while HNMT is present in cytosol of cells and it is able to degrade histamine only in intracellular space [7]. Since DAO actually represents the main barrier of absorption of histamine in blood [7], this research focuses on this enzyme, as well as the following description does.

In mammals DAO is produced mainly in tissues like the small intestine, ascending colon, placenta and kidneys [5]. Particularly during digestion, the DAO enzyme is continuously synthesized in the mucosa of the small intestine [4]. In the intestine, its activity varies depending on its location [1]: it increases progressively from the duodenum to the ileum, located mainly in the intestinal villi [5]; it requires the vitamins B6, C and copper to function properly [1]. DAO can also metabolize other biogenic amines such as putrescine and cadaverine but it shows a preference for histamine [5], both the one introduced with foods and the one formed naturally by the intestinal bacteria [7], in the gastrointestinal system. Moreover, DAO enzyme is involved in other physiological functions including the regulation of the inflammation processes, proliferation, allergic response and ischemia [11].

The evaluation of DAO level is considered for medical purposes, in diagnosis stages since it reflects intestinal damage and repair [12]. Specifically, plasma DAO levels measurments are

recently gaining more interest as non invasive diagnosis tools to evaluate the function of the small intestinal mucosal barrier, as well as gastrointestinal diseases, histamine intolerance (HIT), migraine, and abnormal pregnancy and in the prognosis of tumors [12].

When an impairment of DAO activity occurs, either because of a genetic pre-disposition, or gastrointestinal diseases, or medications with DAO inhibitors the histamine blood level rises [8]: this state is known as DAO deficiency [5]. The conditions bringing to this state are found to be inflammatory bowel pathologies affecting mucosal integrity, chemotherapy, gastrointestinal disorders like carbohydrate malabsorbtion and nonceliac gluten sensitivity, gastroenteritis, irritable bowel syndrome, short bowel syndrome and gastrointestinal surgery [5]. About the DAO inhibition due to interactions with chemicals it resulted that it can be caused by the fact certain drugs are characterized by a chemical structure which is similar to the one of histamine, leading to the binding of the DAO active site [11]. Reduced DAO activity can be also found in patients with chronic renal failure, viral hepatitis, advanced hepatic cirrhosis, chronic urticaria [13] and in all the cases of lack of the cofactors, vitamin B6, vitamin C, copper and zinc [14].

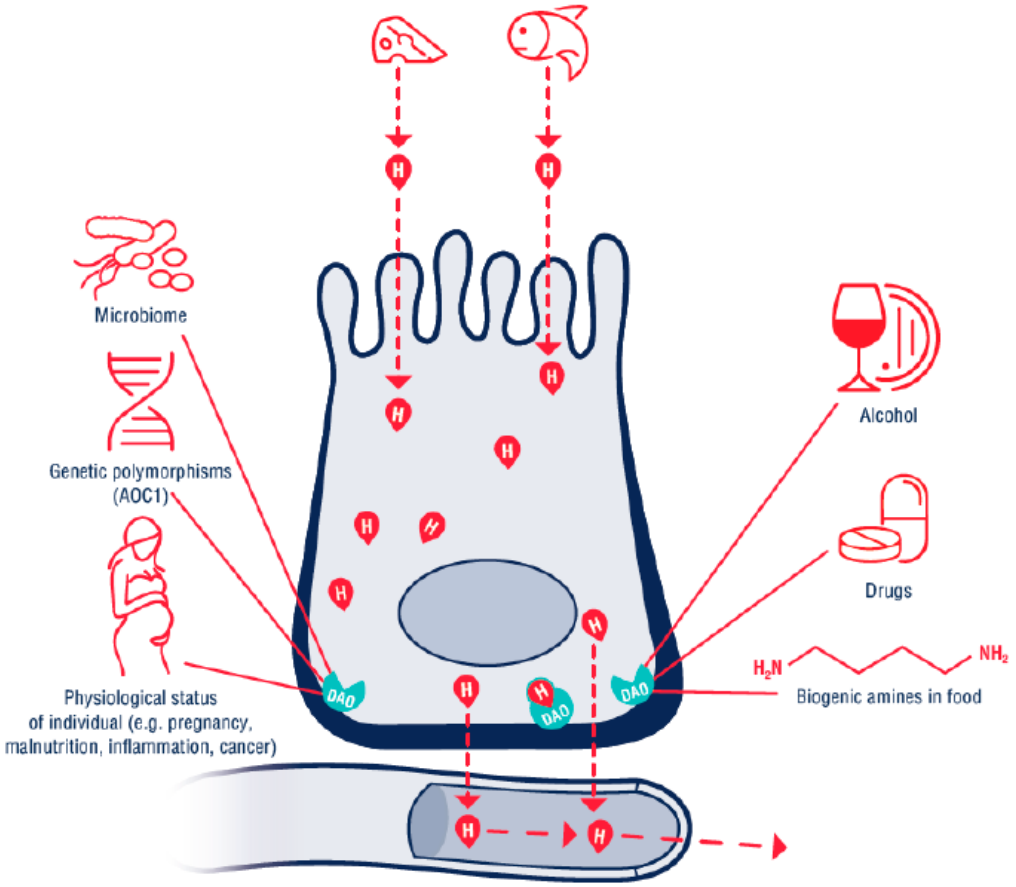

Figure 1-2 *"The function of diamine oxidase (DAO) in the enterocyte. The red drop with H shows the histamine released from food. The histamine passes through the enterocyte into the circulation. The DAO enzyme on the basolateral membrane creates a barrier, and the histamine obtained from the food is metabolized (the red drop with H with a green contour). DAO activity is directly/indirectly dependent on internal and external factors"*[4]

According to the International Society of DAO Deficiency, the effects of DAO deficiency on the organism can be multiple and adverse, affecting the respiratory system (nasal congestion, asthma); the cardiovascular one (hypotension, hypertension, arrhythmias); the central nervous one (migraine, headaches, hangover, dizziness); the digestive one (irritable bowel syndrome, constipation, satiety, stomach pain, vomiting); the muscular one (fibromyalgia, muscle pain) and the skeletal one (osteopathic pain) [15]. In blood tests the reference values of a normal DAO activity are considered higher than 10 U/ml [1].

1.2.1 Histamine intolerance

The histamine intolerance (HIT) condition results from the imbalance between the supply and degradation of histamine [1]. It is recognized as a disorder provoking allergy-like symptoms, that occur even after the ingestion of small amounts of this amine [16]. Food intolerance is also

commonly known as nonallergic food hypersensitivity, as a response caused by a food or its components when ingested in normally tolerated doses by the healthy population [5]. DAO deficiency represents a subgroup of the population affected by HIT [4].

The symptomps occurring after the blood histamine level increases above 1.0 ng/ml are numerous: urticaria, itching of the skin, gastrointestinal disorders, sneezing, rhinitis, headache, arrhythmia and hypotonia [1]. There can be secondary symptoms, which result in an increase of the blood pressure (even though histamine itself causes its decrease) and additional tachycardia, dysrhythmias, nervousness, sensation of inner tremor and sleep disturbances [7].

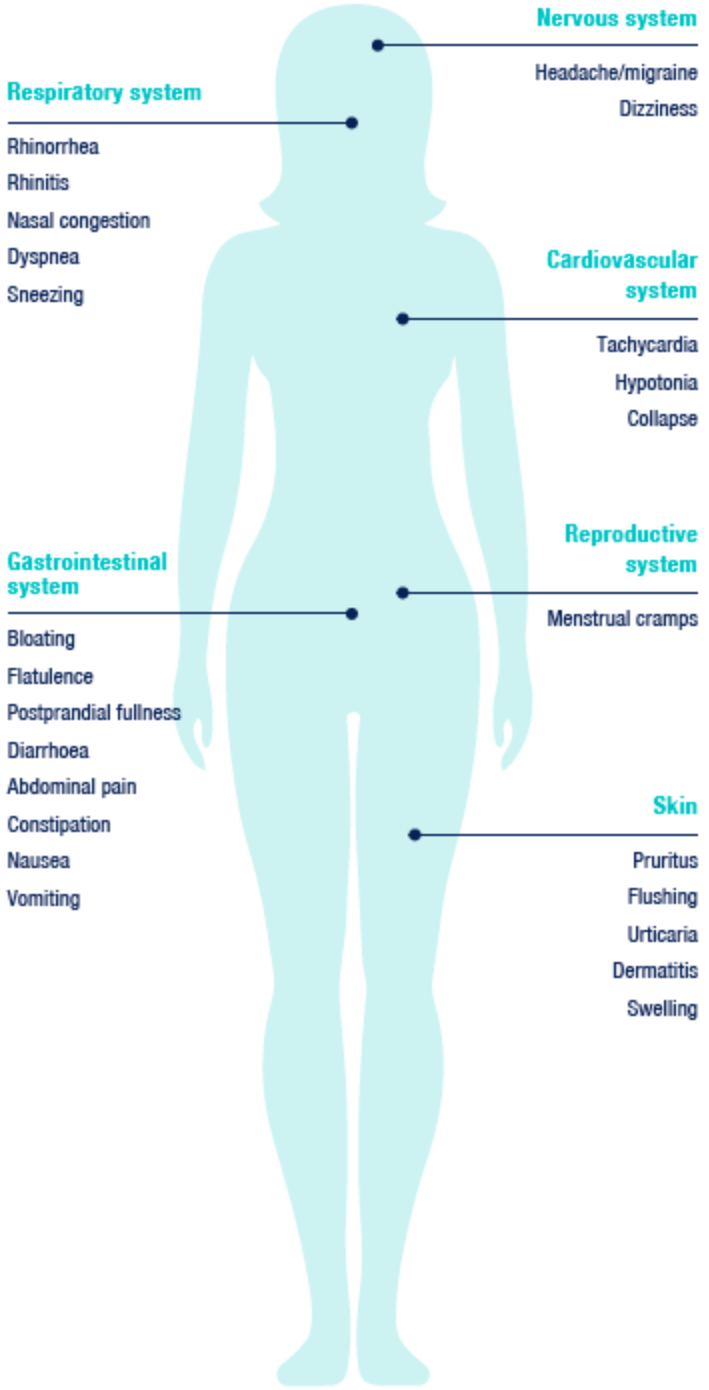

Figure 1-3 *Typical symptoms of intoleration to histamine on a woman* [4].

The difference with allergy is that in this case a single antigen causes the development of symptoms, while the histamine intolerance depends on the ingested cumulative amount [7], and so on the accumulation in blood. Histamine intolerance can develop during time, and it statistically affects mainly middle-aged women; overall it is estimated that 1% of the world population is intolerant [1]. The sensitive to amines population include also individuals with

respiratory and coronary heart diseases, hypertension problems or vitamin B12 deficiency and people with gastrointestinal problems (gastritis, irritable bowel syndrome, Crohn's disease, gastric and colon ulcers) [8]. Generally this kind of health issue is difficult to be diagnosed because of the wide spectrum of symptoms, although it is spread throughout the world [4].

1.2.2 Histamine intoxication

If the detected concentration of biogenic amines raises above 50 g/g, in foods of animal origin like fish, meat, eggs, cheese and in fer-mented foods, chemical poisoning can occur [8]. Specifically histamine poisoning or intoxication can happen when such these histamine-rich foods are consumed in an excessive quantity [1]: fish is considered worldwide to be the most frequent cause [4]. According to the last numerous studies the amount of histamine inducing intoxication symptoms is an extremely variable information, resulting in a range that goes from 100 to 10,000 mg/kg [17]. Histamine toxicity can also be caused by the presence of other biogenic amines since they can provoke the saturation of DAO and HNMTs enzymes, unable to bind and degrade the remaining histamine [4]. For example in certain fermented products (i.e., dryfermented sausages, cheeses, and plant-fermented products) and in semi-preserved fish derivatives there are frequently higher levels of putrescine and/or cadaverine than histamine [17].

From a symptomatic point of view histamine intoxication is characterized by short incubation periods (i.e., 20–30 min post-ingestion), followed by generally low/moderate severity symptoms affecting the skin (e.g., redness, rash, urticaria, pruritus, edema and local inflammation), the gastrointestinal tract (e.g., nausea, vomiting and diarrhea) and the hemodynamic (hypotension) and neurological (e.g., headache, palpitations and tingling) bodily functions, and a consequent recovery after few hours [5]. It is also possible that the intoxication results in a fatal outcome [4].

This study is oriented towards the prevention and cure of histamine intolerance caused by DAO deficiency, for this reason the following section describes all the found approaches to this problem.

1.3 Histamine intolerance/ DAO deficieincy cures

For histamine intolerance due to DAO deficiency there is no specific solution to avoid the HIT effects [12]. "The basic treatment of histamine intolerance caused by DAO is the use of a diet

based on products with a low histamine content" [1]. Generally the diet should consist of fresh foods as much as possible, while processed, preserved, and highly elaborated foods should be avoided [7]. Additionaly, the histamine-free diet can be completed with the administration of food supplements of vitamin C and vitamin B-6, which lead to an increase in DAO activity, supporting the histamine degradation [8]. In a case report it has been shown that the histamine-free diet resulted effective after about 2 months of dieting, reducing symptoms and increasing the DAO level, but due to the exclusion of many foods, it also resulted in malnutrition [1].

An alternative approach is represented by the supplementation with probiotic microorganisms, in particular of strains that do not produce L-histidine decarboxylase that could lead to modulations of microbiome: ideally, these strains would be capable at the same time of degrading histamine (or other biogenic amines) [4]. Clinical trials assessing the effects of probiotic administration as an HIT treatment are not found in literature up to the present, while from experimental trials, it seems that members of Bifidobacterium genus could be considered for the supplementation [4].

Antihistamines can be a solution too, even though they have been shown to have no effect on DAO activity, so it would be better to use them as a symptomatic and short-term treatment [1]. On the other hand, HIT can be effectively cured by taking DAO supplements that would break down histamine in the meals consumed [1]. Commercially available dietary DAO supplements, like Daosin®, are products of animal origin: the enzyme is specifically extracted from porcine kidneys [12]. According to a European Food Safety Authority (EFSA) decision porcine kidney extract could be distributed as nutrition supplement, in doses of 0.3 mg [4]. It was also stated that the maximum daily ingested quantity of this product is 0.9 mg, and that the supplements would have been formulated to be gastro-resistant in order to deliver the active enzyme only once in the small intestine [4]. The effectiveness of the use of DAO supplements was demonstrated in many studies conducted so far, confirming a reduction of at least one typical symptom of HIT in 93% of the respondents [1]. The clear advantage of this method of treatment is the possibility of a less restrictive diet [1]. Anyway, it is known that a very high DAO activity of at least 690 nkat (where one nkat was defined as the amount of enzyme that converts 1 nmol histamine per second at 37 °C, which corresponds to 0.06 Enzyme Units (μmol of degraded histamine per minute)) is required under actual simulated intestinal conditions to degrade food relevant amounts of histamine, and that to obtain this activity, around 1.4 kg pig kidneys would be required for the extraction and partial purification [18]. In contrast to the positive results obtained from DAO supplemets application, the extraction from porcine kidneys is

characterized by an insufficient yield, from an economical point of view [18]. Moreover regarding the DAO activity of porcine kidneys in the commercialized food supplements different results were obtained, in some cases giving specific values ranging from 0.04 to 0.20 mU/mg, in some others stating the complete absence of activity [5]. In conclusion overall these findings coincide with the need to consider alternative sources to porcine kidney DAO for the production of supplements fighting the HIT disorder [5].

For this purpose DAO enzyme of plant origin has been studied and a higher catalitic capacity has been found, with respect to the one from porcine kidneys [5]. Particularly the enzyme extracted from legume sprouts is to be considered having an activity of 230.8 ± 11.9 mU/g that varies depending on the legume species [16]. It has been demonstrated that the step enhancing the degradative ability of the enzyme is the germination of the seeds, particularly a 6 days germination carried out in darkness has shown to provide the maximum histamine-degrading capacity [16]. The vegetal DAO from pea (*L.sativus*) showed also a higher stability in simulated intestinal fluids than what characterizes the free pig DAO: in the first case the half-life period was recorded around 16 hours while in the second case it was of 19 minutes [18]. The use of DAO form plant sources in food supplements would bring also economical and social advantages, since the assumption of this enzyme would regard the vegeterian and vegan pupulation, and the consumers unable to eat porcine derived products because of religious restrictions; moreover this practice would be in accordance with the current call for action of the Sustainable Development Goals [5]. “More recently, vegetal Diamine Oxidase (vDAO) from Pisum sativum was proposed in capsule and tablet forms” [19]. Generally vegetal DAO has been studied for other various therapeutic effects, as it has been shown to possess antioxidant, cardioprotective, and even antitumoral properties [20]. Vegetal DAO application, which represents one of the most recent interests in this field, is being researched and also innovated. For instance, Blemur et al. (2015) immobilized DAO white pea (*Lathyrus sativus*) by inclusion in microspheres formed by “ionotropic gelation of CMS/alginate by complexation with Ca2+”. The application of such an external structure was thought to obtain a more compact and less porous shield, so that the access of the acid gastric fluids was limited [20]. Indeed it resulted that the immobilized enzyme remained active, retaining up to 70% of its initial activity in simulated gastric fluid (pH 2.0) and the 65% of its initial activity in simulated intestinal fluid (pH 7.2) with pancreatin over 24 hours, while the free enzyme was completely inactivated in similar conditions [20].

An alternative approach, to the extraction of DAO from animal or plant sources, is the overexpression of DAO in a suitable microbial host [21]. This is the case of the so called microbial histamine oxidizing enzymes (HOX) that shows a high kinetics of action (*K*m values), and for this reason considered to be a good possibility for the formulation of dietary supplements in tablets form or histamine-reduced foods [18]. The resistance of the enzyme of such an origin was recorded to be higher than the one characterizing the animal DAO, but lower than the one of plant origin DAO, always depending on the amount and type of food ingested: it was shown that the half-life period of a microbial DAO can last up to 30 minutes in simulated intestinal fluids. [18].

As it was eplained, both because of its evident therapeutic contribution as food for medical purposes [4], and because it represents the starting point of studies regarding DAO deficiency cure approaches, DAO of animal origin (from porcine kidneys, indeed) was chosen to be investigated.

The results obtained in this work must be interpreted as a model and a base over which it is appropriate to develop the research in this field.

Chapter 2

Materials and methods

This section includes the steps followed for the enzyme activity analysis, in each considered treatment, starting from the preparation of the samples and all the necessary solutions, ending with the enzyme activity calculation explanation. An in-depth description of the working principle of the used instrumentation is inserted too.

2.1 Preparation of the samples, pH and thermal treatments

As previously mentioned, this study is focused on determining the resistance of porcine DAO to different pH and temperature treatment conditions. The preparation of the samples was the same for both types of treatments.

2.1.1 pH treatments

Samples of 1 ml were prepared according to the following steps. 20 mg of porcine kidney DAO (Sigma-Aldrich, USA) was weighted and introduced into an Eppendorf tube. The tube was filled with 1 ml of a different buffer solution according to the type of treatment. The tube was mixed using a vortex, before the treatment start.

The treatment at different pH conditions consisted in the preparation of five different buffer solutions: pH 3, 5, 7, 9 and a neutral 1,4-Piperazinediethanesulfonic acid, Piperazine-1,4-bis (2-ethanesulfonic acid), Piperazine-N, N'-bis (2-ethanesulfonic acid), commercially known as PIPES buffer solution in which DAO behavior is already known. Each pH condition was conducted in triplicate. The buffer solutions at different pH values were prepared according to Table I. The pH 3, 5 and 7 buffer solutions were prepared by mixing in different proportions a citric acid solution (0.1 M) and a phosphate solution (0.2 M) (both in distilled water). The pH 9 buffer was prepared by mixing a solution of boric acid and KCl in 1 L of distilled water (0.2 M) and a NaOH 0.2 M (in distilled water) solution. The mixing was realized by taking 50 ml of the first and 20.8 ml of the second solution, and by adding distilled water until a volume of 200 ml was reached. Finally the neutral buffer solution was realized by mixing 3.78 g of PIPES,

in 500 ml of distilled water. Once all the buffers were prepared the pH was measured showing actual values of 2.778, 5.141, 7.049 and 8.722. The PIPES buffer solution is naturally acid and therefore pH was adjusted by the addition of a $NaHCO_3$ 0.9M solution, until a 7.0-7.2 pH value was reached.

Table 2.1 *Buffer solutions compositions, for each pH.*

pH	Buffer solution 100, 200 or 500 mL
3	80.3 ml of citric acid 0.1 M + 19.7 ml of phosphate solution 0.2 M
5	49 ml of citric acid 0.1 M + 51 ml of phosphate solution 0.2 M
7	19 ml of citric acid 0.1 M + 81 ml of phosphate solution 0.2 M
9	50 ml of boric acid and KCl solution + 20.8 ml of NaOH solution 0.2 M + distilled water until 200 ml were reached
Control	PIPES solution, 3.78 g of 1,4-Piperazinediethanesulfonic acid, Piperazine-1,4-bis (2-ethanesulfonic acid), Piperazine-N, N'-bis(2-ethanesulfonic acid) in 500 ml of distilled water

When all the samples were prepared (20 mg of commercial DAO in 1 ml of buffer solution) and well mixed using a vortex, they were stored for 1 hour, 7 days and 60 days at 4°C. After the storage the samples were analyzed. In this way, the effect of the storage time at different pH on the DAO activity was analyzed.

2.1.2 Thermal treatments

Once the study of the DAO resistance to different pH conditions was completed, the study of the effect of temperature on the DAO activity was carried out. For that purpose, and as for the pH study, 20 mg of commercial DAO was mixed in 1 ml of the neutral PIPES buffer solution to make one sample. The study of DAO resistance to temperature was divided into two stages: first considering conventional storage temperatures, and later thermal treatment temperatures were investigated. The storage conditions chosen were -18°C and room temperature (23±1°C), while the chosen thermal treatment conditions were 40, 60, 70°C and 80°C. The enzyme activity analysis was performed after 1 hour and 7 days of constant storage conditions, while the thermal treatment times were of 2, 5, 10, 15 and 20 minutes for the treatment at 80° C and 5, 10, 15 and 20 minutes for the treatments at 40°C, 60°C and 70°C, according to the usual industrial practices.

For every pH and temperature treatment condition, a time-zero sample (control sample), was also considered: 20 mg of commercial DAO were mixed in 1 ml of PIPES solution and analyzed straightforward, in order to evaluate the activity of the untreated enzyme and to compare it with the one after the treatments.

2.2 Enzyme activity analysis

The chemical reaction of histamine degradation by diamine oxidase (DAO) is described by Equation 2.1.

$$Histamine + O_2 + H_2O \xrightarrow[\text{DAO}]{} \text{Imidazole-4-acetaldehyde} + NH_3 + H_2O_2 \quad [22] \qquad (2.1)$$

In agreement with the procedure detailed by Ketter et al. (2020), the enzyme activity analysis was based on a colorimetric assay [23]. Specifically, the reaction between hydrogen peroxide and the peroxidase enzyme (POD) was induced according to Equation 2.2: given that the production of methylene blue and the consumption hydrogen peroxide were linked by a stoichiometric proportion it was possible to correlate the color intensity of each sample directly to the activity of the enzyme. The assays were performed with a spectrophotometer, which principles and functions are described in the next section.

$$DA-67^{+} + H_2O_2 + H_{3+}O \xrightarrow{POD} Methylene\ blue^{++} + 2H_2O + 2 - Amino(N-carboxy)sodium\ acetate \quad [24] \quad (2.2)$$

The following explanation describes the steps of the enzymatic activity analysis.
Firstly, the reactant solutions were prepared: 0.0036 g of histamine dihydrochloride, 0.001 g of 10-(Carboxymethylaminocarbonil)-3,7-bis(dimethylamino) phenothiazine Sodium Salt (commercially called DA-67) and 0.0052 g of horse radish grade I peroxidase (commercially named POD) in 20, 50 and 5 ml of PIPES solution respectively. The PIPES solution was prepared as explained in section 2.1. The PIPES solution is the neutral buffer solution considered to be the ideal environment for the histamine degradation by DAO (Equation 2.1). A buffer solution is needed in order to let the system to maintain the pH at the established value, since the chemical reaction of histamine degradation causes a change in the pH. Once the reactant solutions were prepared they were stored in darkness at refrigerated conditions (4 ºC).

The tests and references were then prepared into 1.6 ml Eppendorf tubes: each test was related to a reference containing the same components of the test minus the histamine. In the reference Eppendorf tubes none of the reactions occurred: the reference enzymatic activity was considered in order to evaluate the relative DAO activity, as explained in section 2.4. Both tests and references were prepared in triplicate, as it follows. All tests were firstly filled with 750 µl of histamine solution, then all the references were filled with the same volume of PIPES solution. Secondly in both tests and references 726 µl of DA-67 solution was introduced, then the Eppendorf tubes were put into a bath at 37°C for 10 minutes. Subsequently, in both tests and references 24 µl of POD solution and 50 µl of a DAO solution were injected and a 10 min bath at 37°C was set again. The addition of 50 µl of sodium diethyl solution (3.38 g of sodium diethyl in 500 ml of distilled water) in both tests and references stopped reactions in Equation 2.1 and Equation 2.2. After a 3 minutes centrifugation at 9669 rpm and 20°C the spectrophotometer analysis came.

2.3 The spectrophotometer [25]

The working principle of the spectrophotometer is based on the ability of natural substances of absorbing the incident energy in form of light. The absorbed energy transfers into the atomic structure in terms of electronic transitions and vibrational and rotational phenomena [25]. Electronic transitions manifest in different ways in both atoms and molecules. Into atoms they occur as jumps of electrons from the ground state of energy (namely their original state) to an excited state which is usually unstable: the excited electrons will lose the acquired energy by reverting rapidly to the ground state [25]. Into molecules the energy transition is more likely to occur at bonds [25]. Each jump and transition, caused by the absorption of energy in form of electromagnetic radiation, is stimulated by a specific wavelength radiation such that there is a relationship, known as specific absorption, between the wavelength of the radiation and the stimulus that it provokes [25]. For a wide range of wavelength the spectrophotometer analysis of any complex chemical structure results in a plot of the absorption “peaks”, or maxima, called absorption spectrum [25].

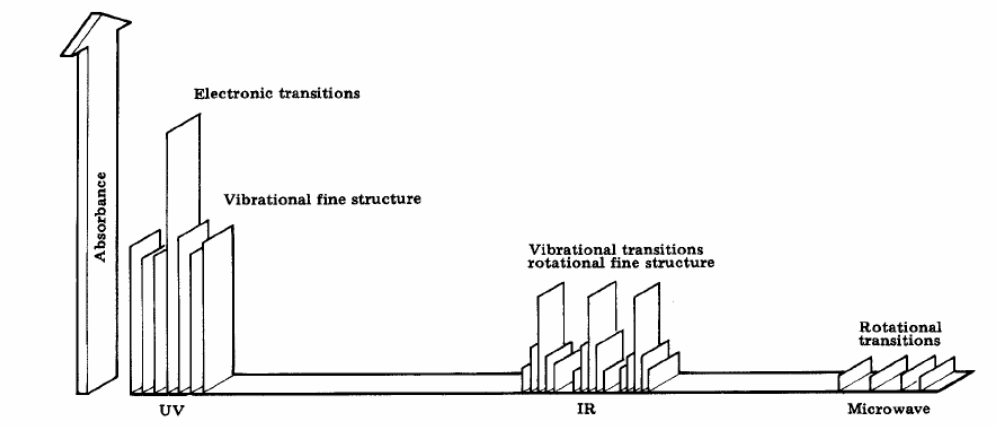

Figure 2-1 *Picks of absorbance* [25].

The instrumetation inlcuded in a spectrophotometer is made of a source of radiation of appropriate wavelengths, a means of isolating light of a single wavelength and getting it to the sample compartment (monocromator and optical geometry), a means of introducing the test sample into the light beam (sample handling) and a mean of detection and measurment the transmitted light intensity [25]:

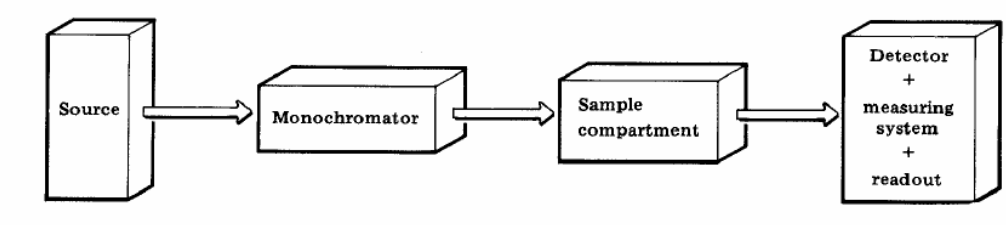

Figure 2-2 *Schematic reprentation of the components of the spectrophotometer* [25].

In this study a Biochrom EZ Read 2000 spectrophotometer was used, having the following characteristics: a tungsten halogen lamp source, a wavelength range of 340-800 nm monochromator, a maximum of 96 cuvettes plate samples compartment and a two silicon diodes detection system [26]. Each cuvette had a capacity of 200 µl so this volume was extracted from each test and reference tube so that eight test and eight reference cuvettes were obtained from the triplicates.

The measurement mode could be chosen between the single wavelength, the dual wavelength, the multiple wavelength and the wavelength scan. In this case the single-wavelength (668 nm) mode was set.

The solution in each cuvette is characterized by a blue color that varies in intensity according to the methylene blue concentration (Equation 2.2). The absorbed fraction of the incident light is the one having the complemetary colour of the one characterizing the solution:

Figure 2-3 *Complementary colours: the upper row represents the solution colour, the bottom one represents the fraction of light absorbed by the solution* [25].

While the transmitted light defines the absorbance, in agreement with the Beer and Lambert law, as follows:

$$A = -\log_{10}\frac{P}{P_0} = abc \tag{2.3}$$

where A is the absorbance, P corresponds to the radiant transmitted power (formerly called the intensity) and P_0 is the radiant power striking the photocell so the intensity of the incident light [25]. The right side of the equation reports a correpsonding to the absorptivity (formerly called the extinction coefficient), b corresponding to the length of the beam in the absorbing medium and c corresponding to the concentration of the absorbing species [25]. In this case c is the hydrogen peroxide concentration and the proportionality constants in the equation are found experimentally through a calibration procedure.

2.3.1 The calibration procedure

A total of six hydrogen peroxide solutions of determined molar concentrations (0, 2, 4, 6, 8, 10 nmol/ml) were prepared, in 1.6 ml Eppendorf tubes. 750 µl of PIPES buffer solution and 726 µl of DA-67 solution were firstly mixed together. After a bath of ten minutes at 37°C 24 µl of POD and 50 µl hydrogen peroxide solutions were added. A second bath was set for 10 minutes

at 37°C, so that reactions described in Equation 2.1 and 2.2 were started. The reactions were then stopped with the addition of 50 µl of sodium diethyl solution. The spectrophotometer analysis followed the centrifugation (20°C, 9669 rpm for three minutes): the single wavelength mode was set on 668 nm. For each test, so for each hydrogen peroxide concentration value, a value of absorbance was obtained and the following linear mathematical correlation was derived:

$$A = 0.000247999\ \mathrm{c} + 0.011580632 \tag{2.4}$$

This linear equation was used to correlate the absorbances to the hydrogen peroxide molar concentrations of the tests performed on the treated DAO: the adopted procedure for the enzyme activity calculation is explained below.

2.4 Enzyme activity calculation

The spectrophotometer work was associated with Galapagos sofware (version 1.3.0.0) through which the measurments were started, the settings were defined and the A results were given, for every analysed cuvette.

As already mentioned, a total amount of eight cuvettes was prepared for every pH or temperature treatment, and for the control, and other eight for the references so that each test cuvette was associated to its reference. The absorbances of the tests and references were measured, and the difference between the two was calculated first. Then, the hydrogen peroxide concentration (referred to the cuvette volume of 200 µl) resulted from the application of Equation 2.4 where A is the absorbance calculated from the difference between the test and the reference measured ones. A total amount of eight concentration values were found, for each treatment and the control. The enzyme activity in U (µmol of degraded histamine/minute) was calculated for each of the eight values, and referred to 1 ml of volume of the DAO samples. The steps are described below.

The molar concentration of the produced hydrogen peroxide resulted to be the same molar concentration of the degraded histamine, because of the stoichiometry of the reaction in Equation 2.1: the amount of degraded histamine in nmol in one reactor volume (so in 1.6 ml) was calculated by multiplying the found concentration by 1.6. In order to know the degraded

histamine concentration in 50 µl of volume, so in the volume of DAO solution injected into each reactor (so into each Eppendorf), the just found amount of histamine in nmol was divided by 0.05 ml. In order to know the amount of degraded histamine in 1 ml of volume, so in the DAO samples volume, this last result was multiplied by 1 ml, since the injected 50 µl of enzyme solution were taken from the 1 ml DAO samples. Finally, the enzyme activity in U was found by dividing this last result by 10^3 to allow the conversion into µmoles and also by the time of reaction in minutes, which is equal to 10 as already explained.

For each attempt an average enzyme activity was calculated between the eight obtained U values, while the residual enzyme activity was found by dividing each averaged U value by the control average enzyme activity. The resulting residual enzyme activity value was expressed in percentage form considering that the control enzyme activity was the 100%, meaning a determined sample gained or lost activity with respect the untreated one. For each treatment final average and a standard deviation were calculated between the U values, and the residual enzyme activities of the three replicates.

2.5 Statistical analysis

The Statgraphics 19 program was used in order to carry the statistical analysis. Particularly simple and multiple factor ANOVA, based on LSD Fischer's tests, were performed on all the collected data. The interaction analysis was done too.

The goal of the ANOVA tests was to individuate groups per each factor, and to understand significant differences among the groups. Significant differences between groups were found when the value of the *p value* resulted smaller than 0.05: in all these cases results are reported and commented in the following sections. The analysis of interactions was carried with the objective of evaluating dependencies between factors: two or more factors were considered dependent on each other if the effect of one factor changed depending on the level of the other factor.

Chapter 3

Results and discussions

In this section, the obtained results are reported for each treatment. Further observations regarding future research and possible industrial applications are included.

3.1 DAO resistance to pH

As explained in section 2.1.1 the commercial DAO samples were treated at different pH values of buffer solutions at 4°C for 1 hour, 7 days and 60 days. The following figures report the results: for each treatment the DAO activities expressed in U (µmol of degraded histamine/min) and the residual DAO activities (in percentage) were found by averaging the results of the three replicates; standard deviations were calculated from the triplicates as well.

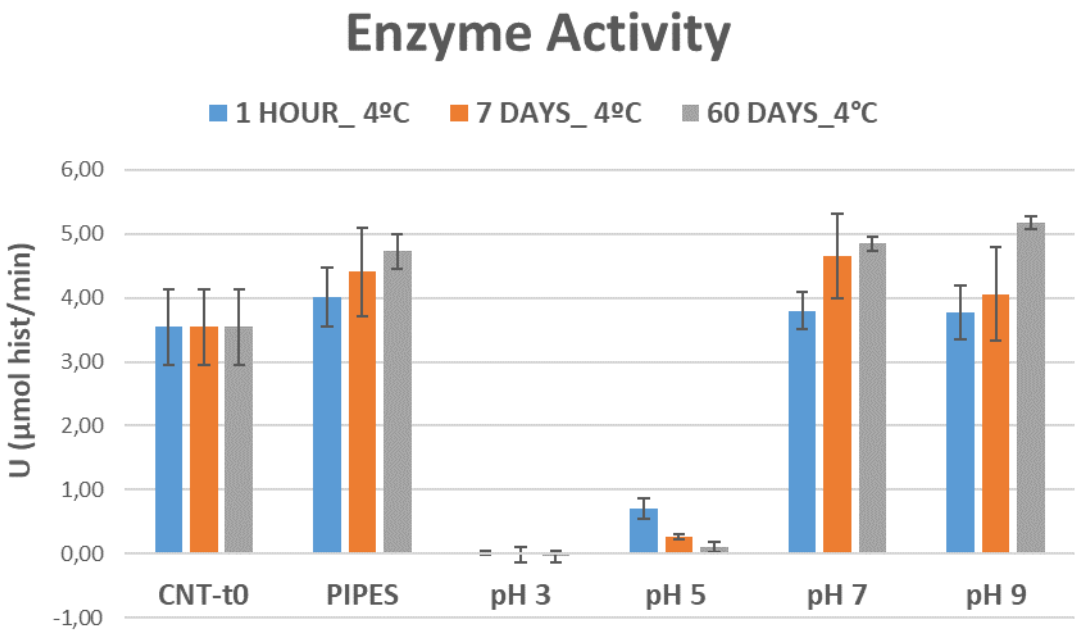

Figure 3-1 *Enzyme activity-resistance to pH.*

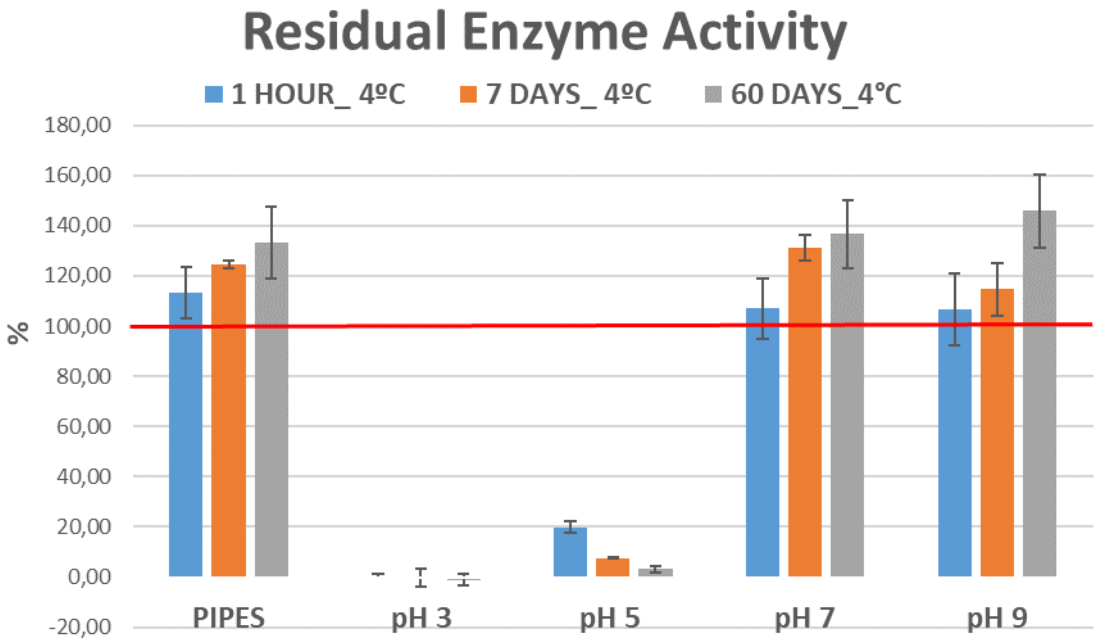

Figure 3-2 *Residual enzyme activity-resistance to pH.*

First of all, it is possible to observe that the commercial DAO generally does not show activity in acid environments (Figure 3-1, Figure 3-2). Specifically in the pH 3 buffer solution the enzyme is not active and this state does not change during time while in the pH 5 buffer solution the enzyme shows activity that decreases during time (0,71 U, 0,27 U and 0,11 U).

Regarding the neutral pH buffer solutions, both the PIPES (pH 7.2) and the pH 7 ones, and the basic buffer solution (pH 9), it can be observed that the enzyme shows the highest values of activity and that they increase during storage time. Therefore, in neutral and basic environments the enzyme activity is enhanced when kept at these pH conditions.

Concerning the commercial DAO residual activity, it is possible to say that the results are the same of those shown for the enzyme activity.

From the analysis of the commercial DAO resistance to pH it is possible to conclude that the most favorable environment is the neutral-basic one and that better activities are obtained after longer storage times (up to 60 days, at 4°C).

About the statistical analysis, the multiple factor ANOVA test compared the enzyme activities by grouping data by pH values and by storage time, in order to study the significant differences. The reported results of the LSD Fischer's test are shown in Figures 3-3 and Figure 3-4:

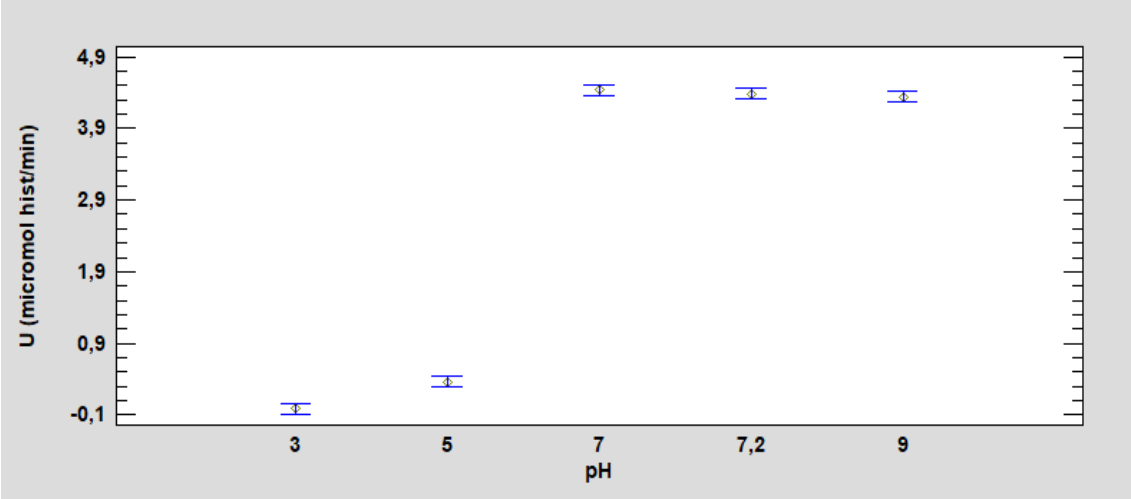

Figure 3-3 *ANOVA test-pH grouping.*

Regarding the pH effect, it is possible to observe that three significantly ($p<0.05$) different groups are found for pH 3, pH 5 and pHs 7,7.2 and 9.

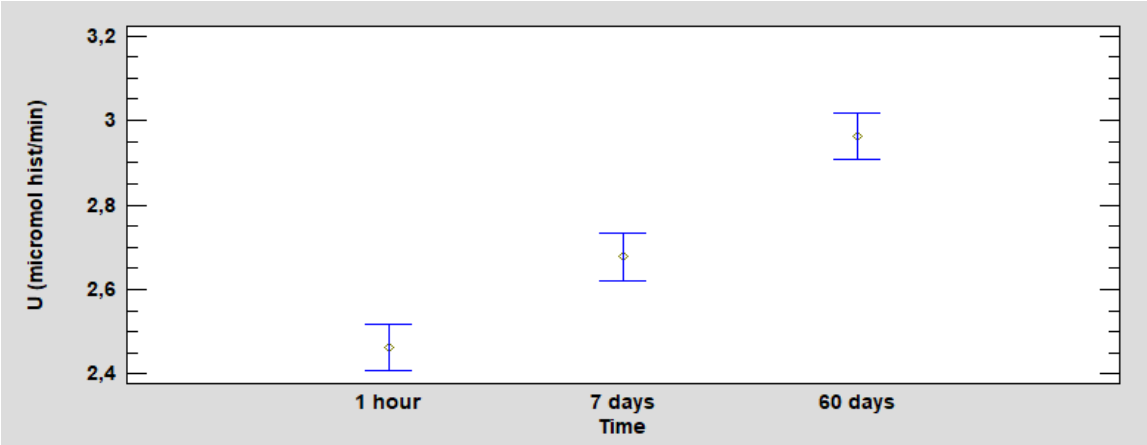

Figure 3-4 *ANOVA test-time grouping.*

Regarding the storage time, it is possible to observe that three significantly ($p<0.05$) different groups are found as well for 1 hour, 7 days and 60 days of time.

From these results it can be said that the enzyme activity of DAO does change significantly if the environment pH changes from values like 3 to 7 and that the enzyme activity does also change significantly according to different storage times, from 1 hour to 60 days, at 4°C. Then it can be concluded that both pH (from acidic to neutral pH values) and storage times (at 4°C) can be considered significant factors.

The interactions graph is reported too, in order to see if the effect of a factor (pH or storage time) affects the changes of the other factor.

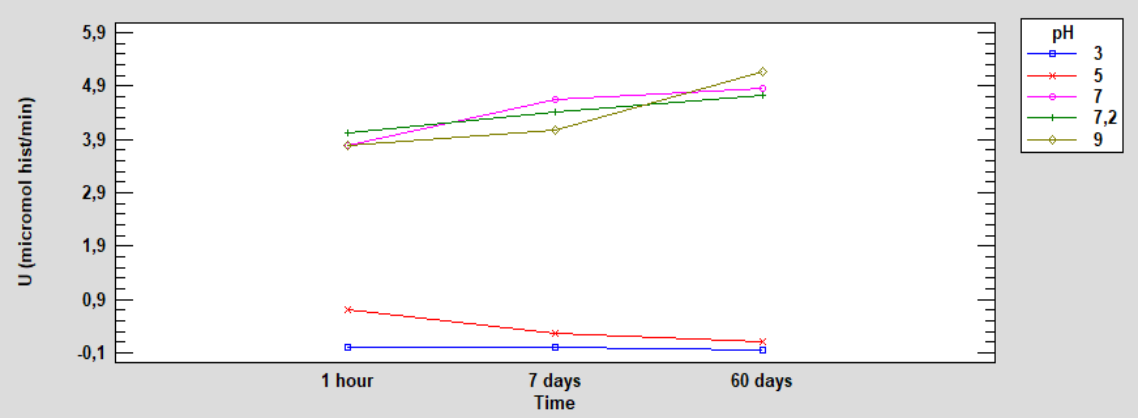

Figure 3-5 *Interaction graph.*

It is possible to observe that, overall, there is an interaction between factors. Specifically, by dividing the pH values in two groups it is possible to say that, even though the pH 3 trend is just slightly decreasing and the enzyme cannot be considered active at this level, at acid pH the time is affecting the activity decreasing it. What happens for the neutral and basic pH values is that the overall trend of the lines results to be positive meaning that the enzyme is more active as storage time increases. Therefore no interaction is detected within the acid pH and the basic pH groups, meaning that the effect of time is the same among different acidic pH values or different basic pH values. Anyway considering all the pH levels there is no shared ranking: DAO activity does not follow an order along time levels, between all the pH cases so as a conclusion to this study it is possible to say that the two considered factors namely storage time (at 4°C) and pH are not independent between each other.

The simple ANOVA test was carried considering only the 7.2 pH (PIPES), in order to study the effect of time (at 4°C storage conditions) on the activity of the enzyme, starting from the untreated sample so the time-zero one.

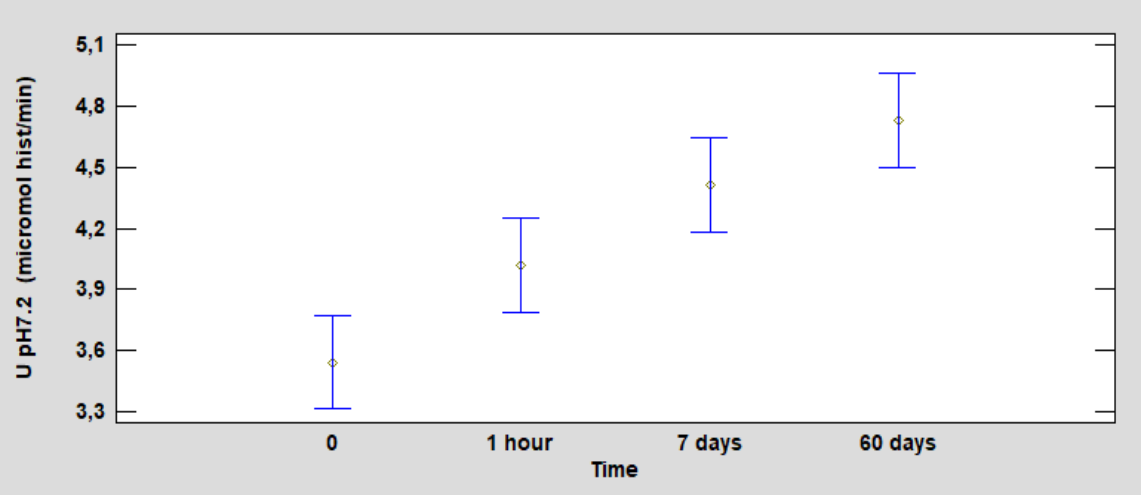

Figure 3-6 *one way ANOVA test-pH 7.2 sample.*

A significant ($p<0.05$) difference is seen between the untreated samples, the 1 hour and the 60 days samples, while there is no significant difference between the 1 hour and 7 days samples and the 7 days and 60 days samples.

Overall, it can be said that at this pH condition the enzyme resistance increases with storage time at 4°C and in particular that the increase is of 33.28%.

From these experiments it has been observed that the neutral PIPES buffer provides one of the highest levels of enzymatic activity, therefore this buffer (and the associate pH condition) was chosen to study the resistance of DAO to temperatures.

3.2 DAO resistance to temperature – storage conditions

As mentioned in section 2.1.2, the storage experiments were carried out maintaining the samples of commercial DAO in the PIPES buffer solution at -18°C and 23±1°C (room temperature) for 1 hour and 7 days. The following figures report the results: for each treatment the DAO activities expressed in U (µmol of degraded histamine/min) and the residual DAO activities (in percentage) were found by averaging the results of the three replicates; standard deviations (represented as well in the graphs) were calculated from the triplicates as well.

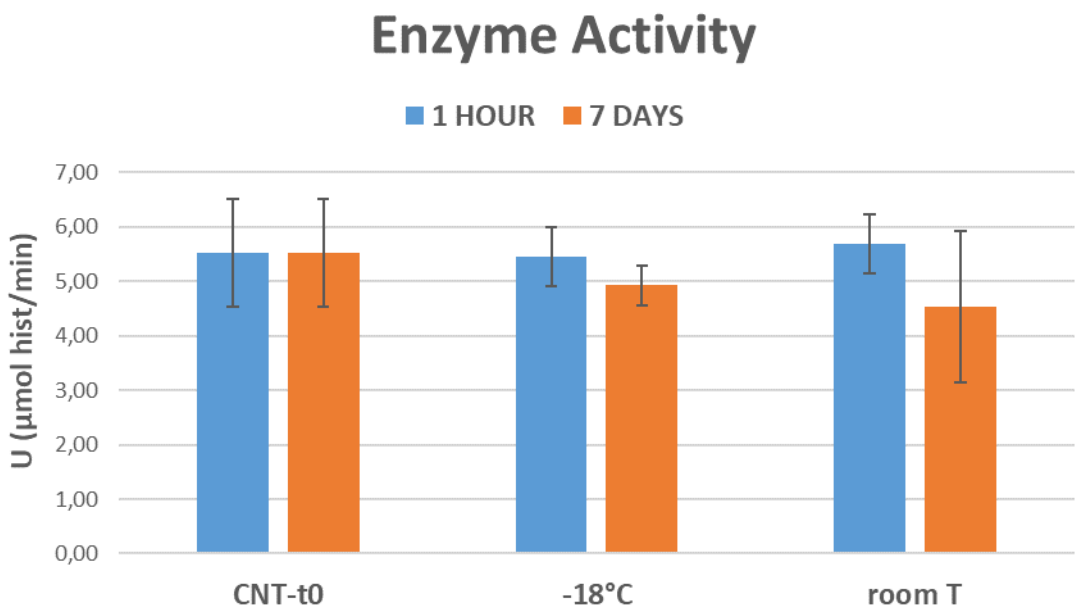

Figure 3-7 *Enzyme activity-storage conditions resistance.*

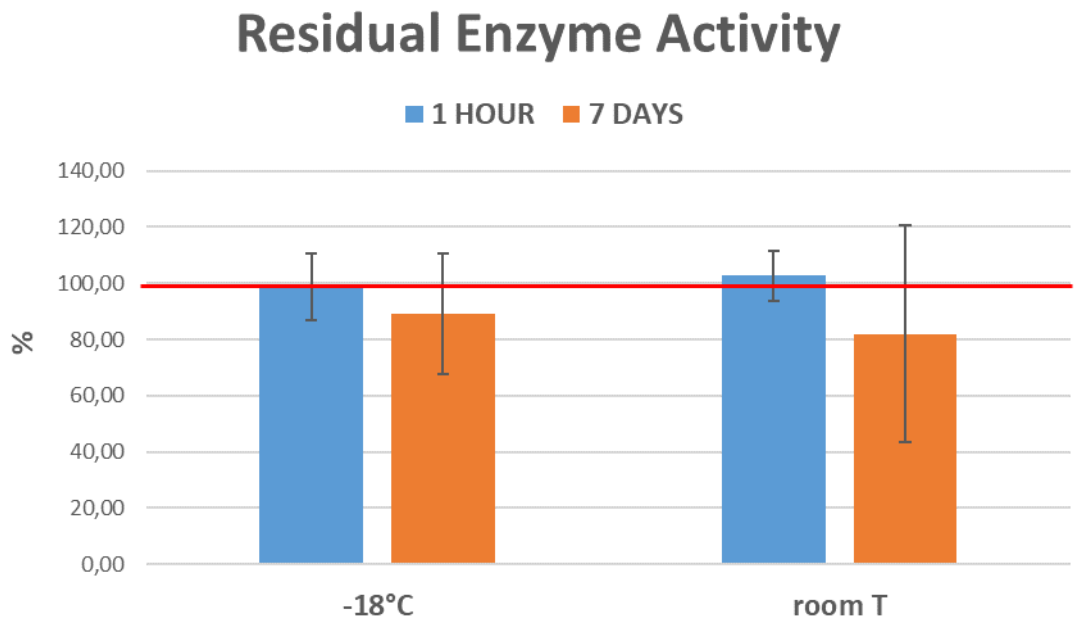

***Figure* 3-8** *Residual enzyme activity-storage conditions resistance.*

It is possible to observe that commercial DAO activity decreases (9.77%) during time, from 1 hour to 7 days of storage at -18°C, anyway the variability results to be large enough to affirm that the differences between the two times are not to be considered significant.

A similar phenomenon occurs at room temperature storage conditions, in this case the decrease in activity from 1 hour to 7 days is higher than at -18°C namely 20.19%.

Generally, commercial DAO activity is maintained after one hour of storage at both temperature conditions; the residual activities calculated with respect to the untreated control sample are close 100%. Moreover, it can be said that the enzyme loses some activity if stored for 7 days of time at both temperature conditions.

A multiple factor ANOVA test was carried out to compare the enzyme activities by grouping data by thermal storage conditions and by storage time, in order to study the significant differences. The reported results are the ones of the LSD Fischer's tests: no significant differences ($p>0.05$) were found between temperature conditions meaning that storage temperature is not an impacting factor on the activity on DAO.

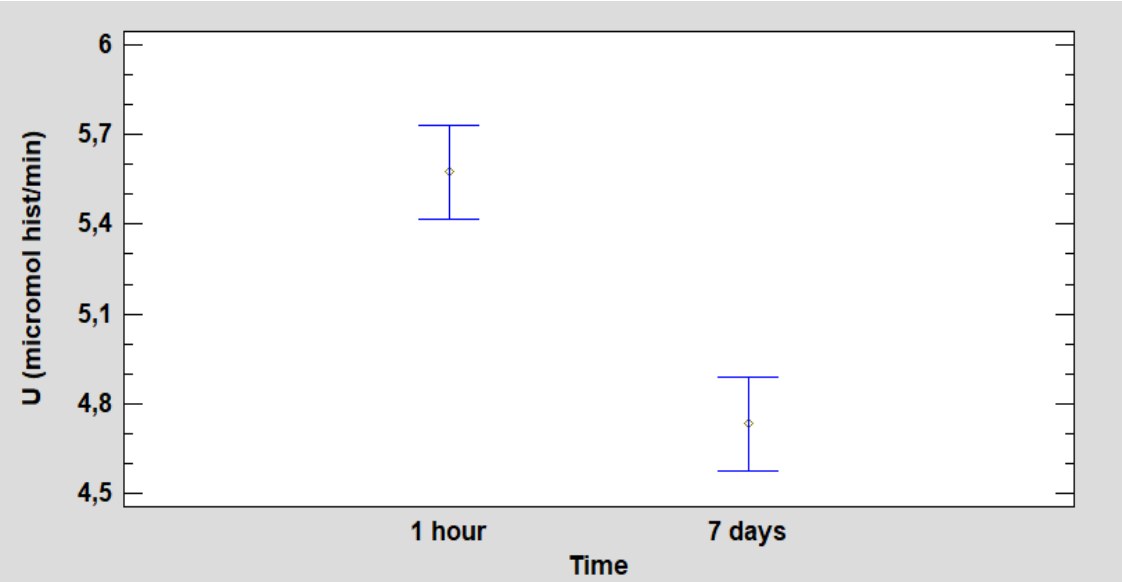

Figure 3-9 *ANOVA test-time grouping.*

Concerning the storage times a significant difference is visible between them two: the enzyme resistance decreases being affected by this factor.

The interactions graph is reported too, in order to see if the effect of a factor (storage temperature or storage time) changes at a changing of the other factor.

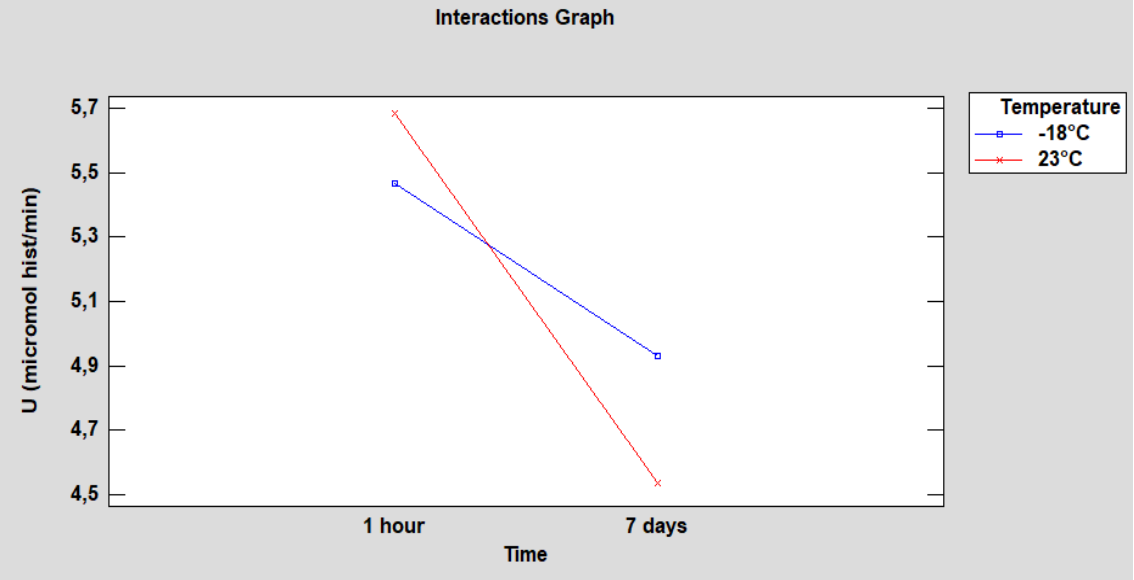

***Figure* 3-10** *Interaction graph.*

The graph shows a simple interaction between the factors: a specific ranking is visible so it can be stated that one storage time is better than the other in all the cases, meaning that storage temperature and storage time are independent factors between each other, and that the change in one factor does not determine a change in the other one.

The simple ANOVA test was carried between the control (untreated) sample data and the two families of samples, namely -18°C and room temperature storage conditions, separately. The goal of this study is to find the effect of the storage (and of the storage time) at different temperatures on the DAO activity. According to the results obtained no significant difference was found between the control sample enzymatic activity and the two storage temperatures one: the enzyme keeps its original activity if stored at one of the two conditions of temperature.

After these analysis, the most favorable storage conditions for the commercial DAO to perform in its histamine degradation activity are decided to be the room temperature conditions in neutral pH environments.

3.2 DAO resistance to temperature – thermal abuse and thermal treatment conditions

Concerning the thermal treatment conditions, experiments were carried out by maintaining the samples of commercial DAO in the PIPES buffer solution at 80° C for 2, 5, 10, 15 and 20 minutes and at 40°C, 60°C and 70°C for 5, 10, 15 and 20 minutes. The following figures report the results: for each treatment the DAO activities expressed in U (µmol of degraded histamine/min) and the residual DAO activities (in percentage) were found by averaging the results of the three replicates; standard deviations (represented as well in the graphs) were calculated from the triplicates as well.

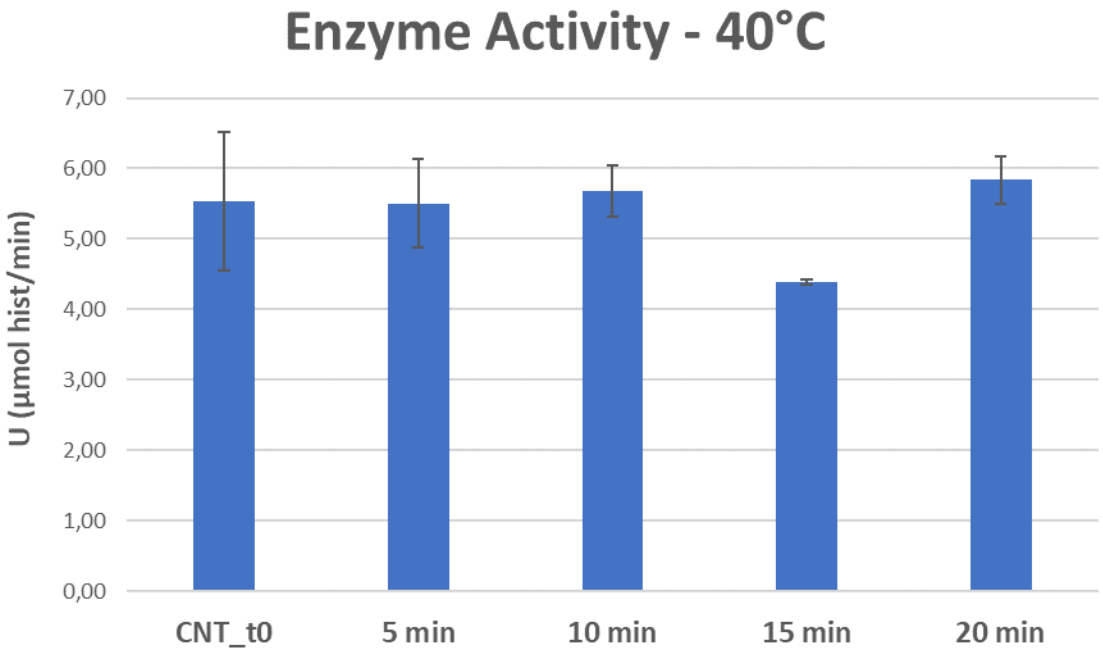

Figure 3-11 *Enzyme activity-40°C thermal treatment resistance.*

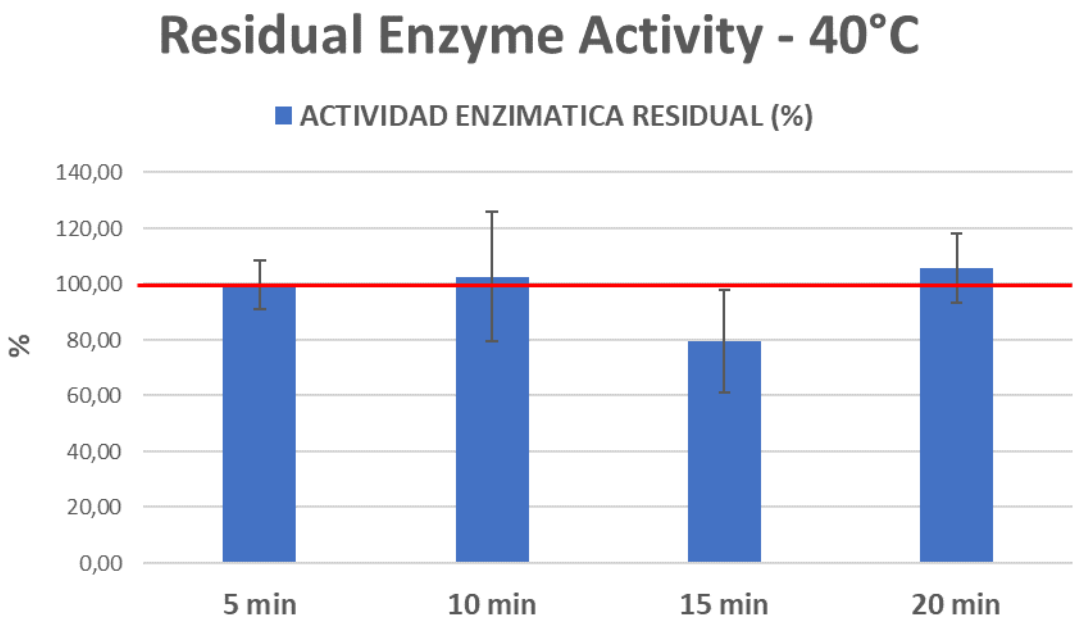

Figure 3-12 *Residual enzyme activity-40°C thermal treatment resistance.*

It is possible to see that the 40°C thermal treatment basically maintains the original activity of the enzyme.

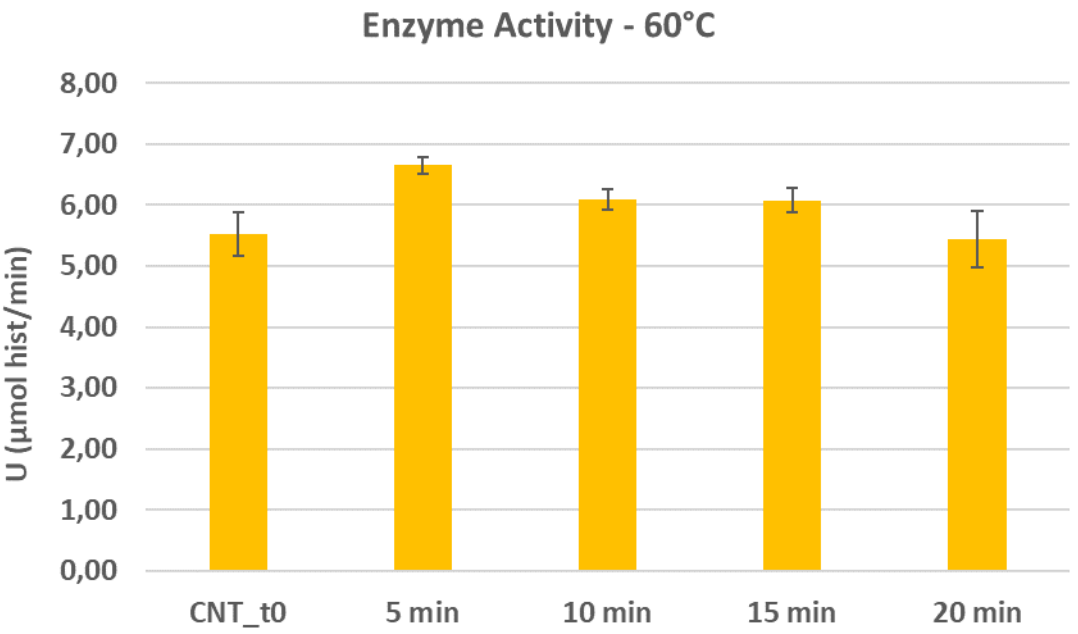

Figure 3-13 *Enzyme activity-60°C thermal treatment resistance.*

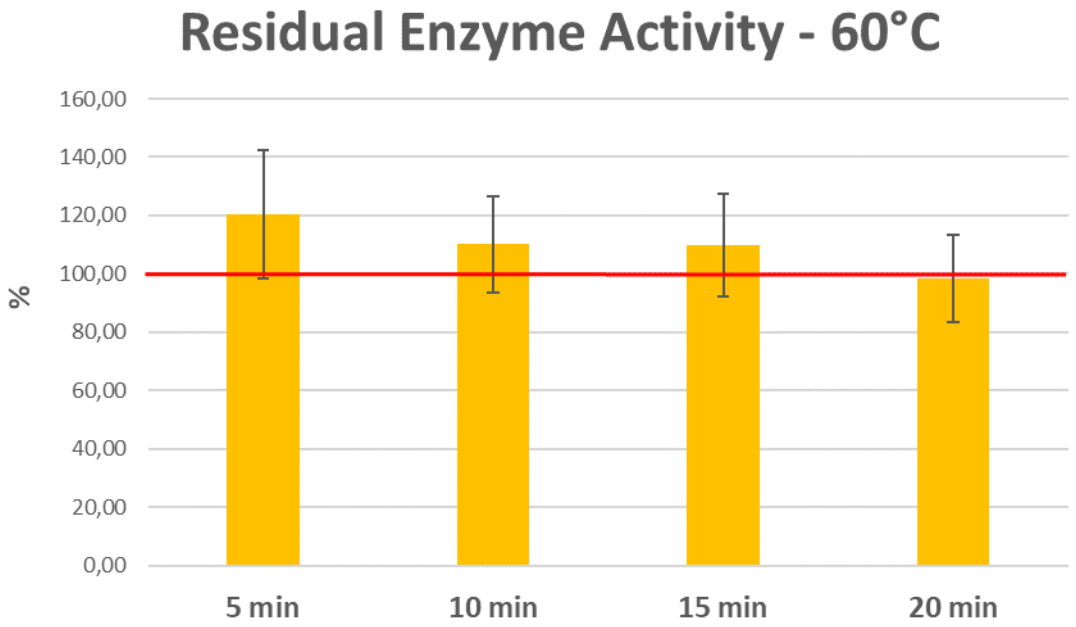

Figure 3-14 *Residual enzyme activity-60°C thermal treatment resistance.*

At 60°C DAO activity is also maintained high, close to the level of the time-zero sample. A slight decrease is observed if the treatment lasts up to 20 minutes, but overall it is possible to say that this temperature can guarantee the preservation of the enzyme.

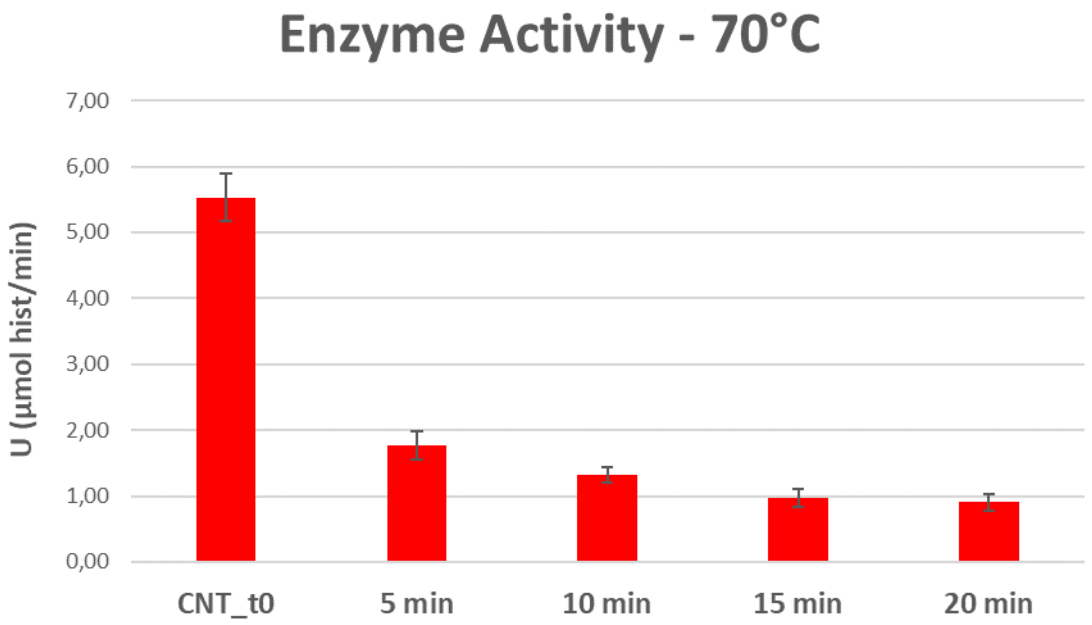

Figure 3-15 *Enzyme activity-70°C thermal treatment resistance.*

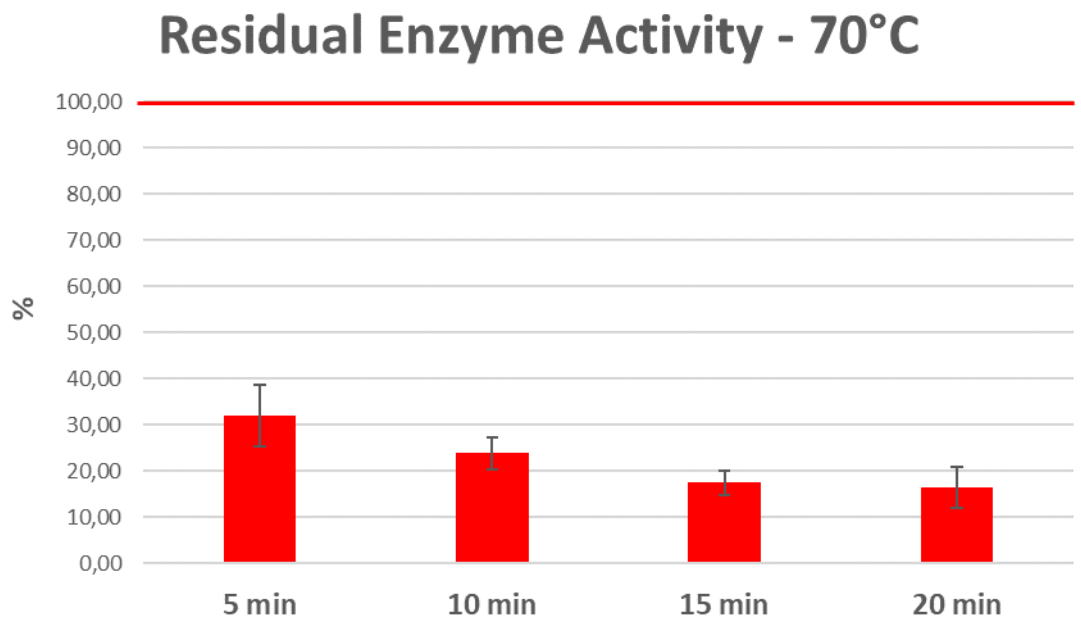

***Figure* 3-16** *Residual enzyme activity-70°C thermal treatment resistnce.*

A different trend is visible for the 70°C thermal treatment: the enzyme activity is reduced just after the first 5 minutes of treatment (a 66.67% decrease is observed with respect to the untreated sample) and it continues decreasing as the treatment is prolonged in time.

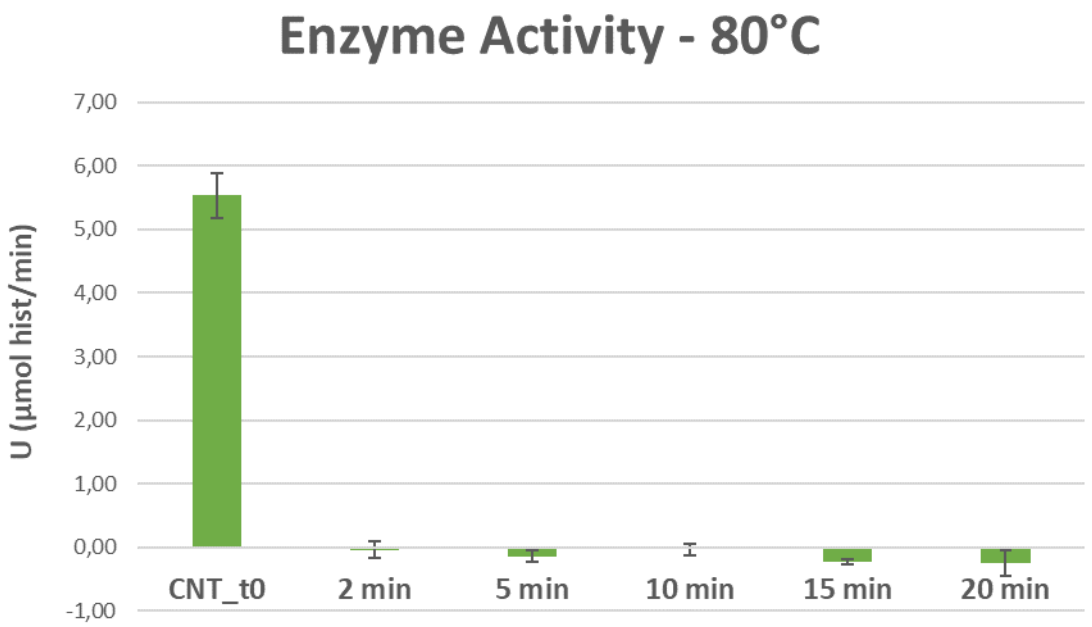

Figure 3-17 *Enzyme activity-80°C thermal treatment resistance.*

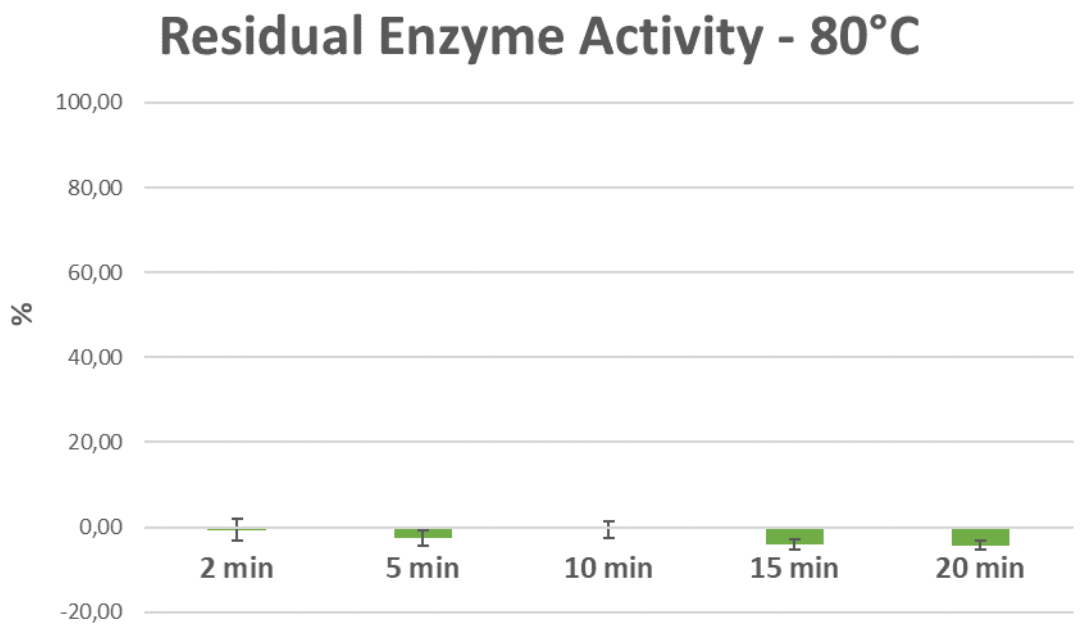

***Figure* 3-18** *Residual enzyme activity-80°C thermal treatment resistance.*

Lastly, no activity is registered in the 80°C thermal treatment at any of the times considered. It is assumed that hypothetical higher temperatures bring to the same results.

For a complete graphical vision of the effect of the different temperatures and times the following pictures are reported:

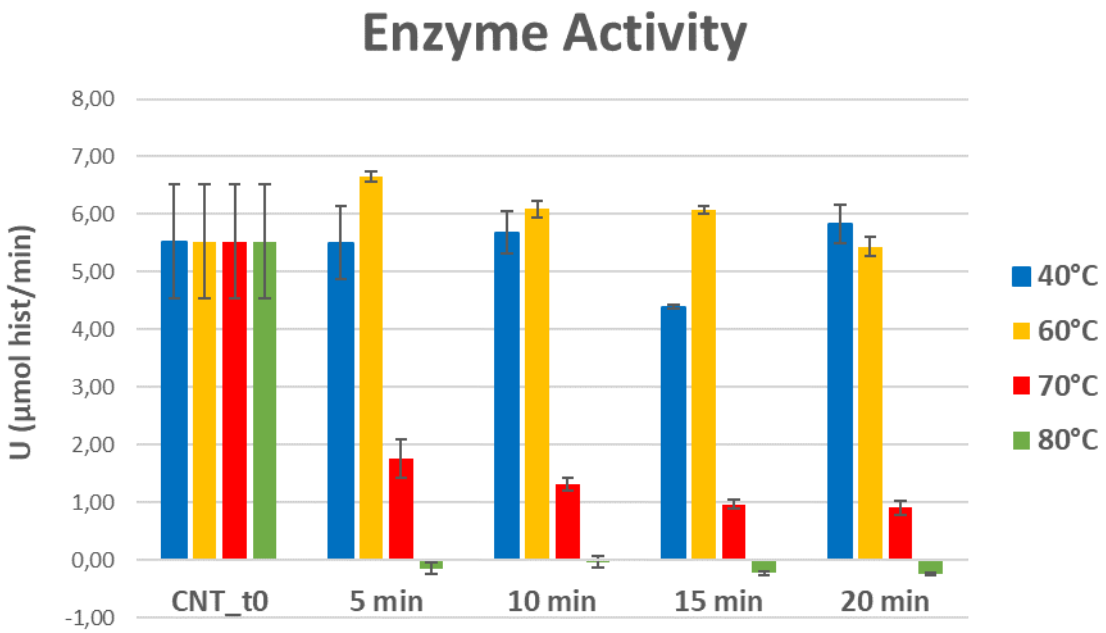

Figure 3-19 *Enzyme activity-thermal treatments resistance.*

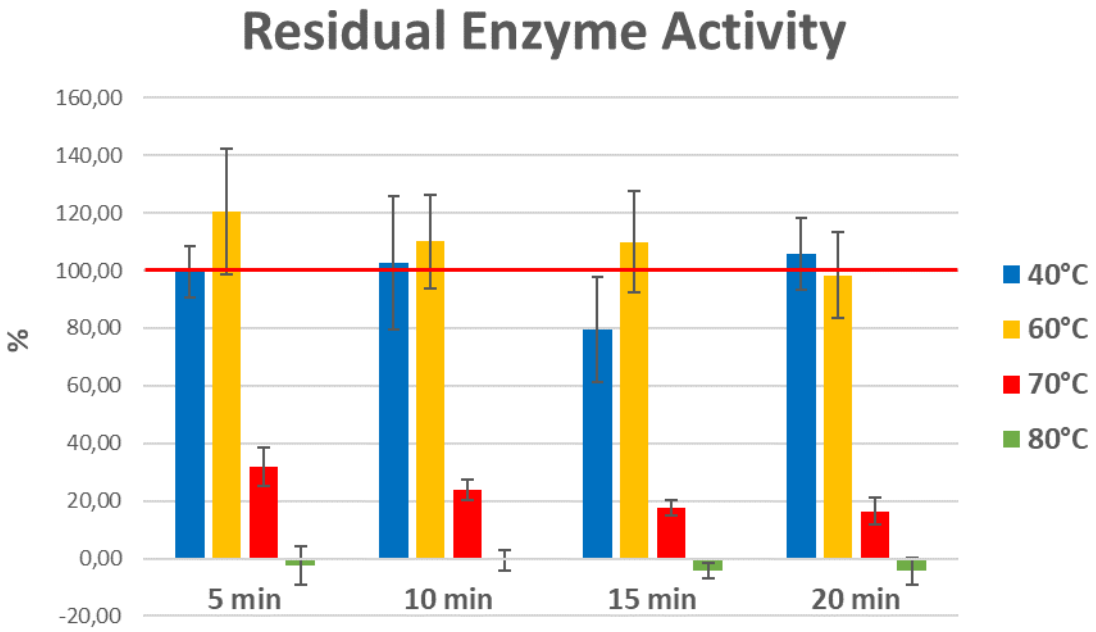

***Figure* 3-20** *Residual enzyme activity-thermal treatments resistance.*

Statistical analysis such multifactorial and simple ANOVA tests were carried to compare the enzyme activities by grouping data by thermal treatment conditions and by treatment time, in order to study the significant differences. The reported results are the ones of the LSD Fischer's tests:

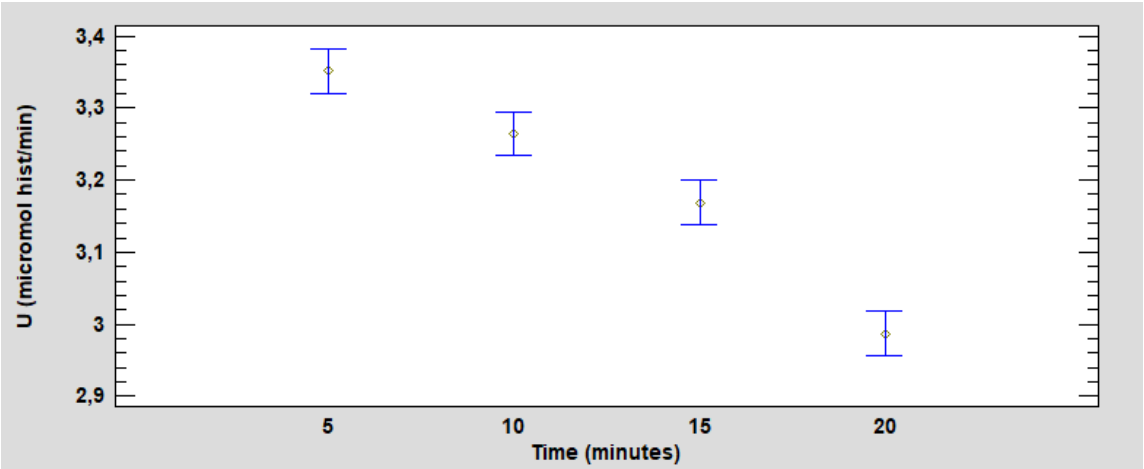

Figure 3-21 *ANOVA test-time grouping.*

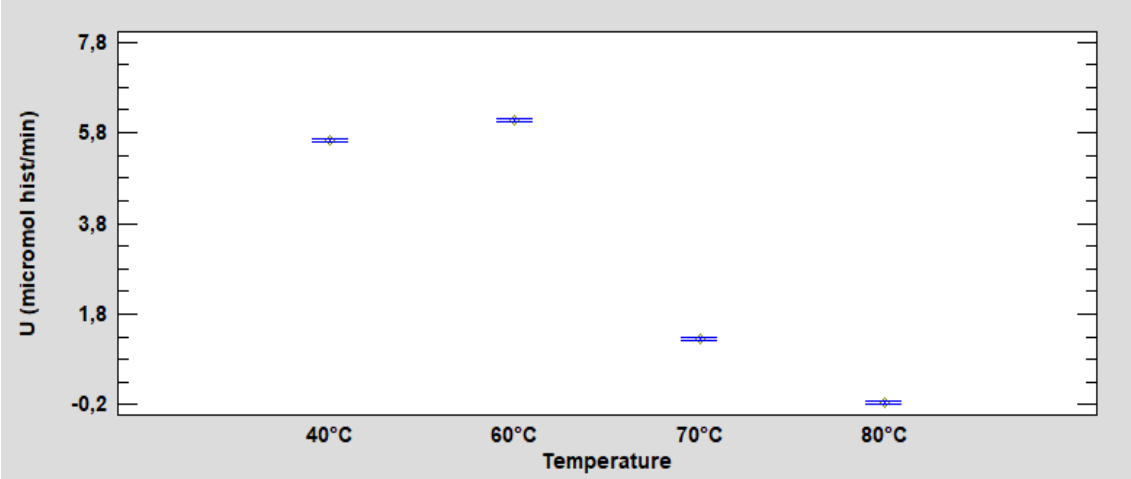

Figure 3-22 *ANOVA test-thermal treatments grouping.*

It is possible to observe that there is a significant difference between all the groups of data, meaning that both thermal treatment times and temperatures affect DAO activity, in the ranges of times and temperatures considered. In particular both increasing time and temperature decrease the activity: a 10.75% decrease from 5 to 20 minutes, and an almost 100% decrease from 40°C to 80°C are detected.

The interactions graph is reported too, in order to see if the effect of a factor (thermal treatment temperature or time) changes at a changing of the other factor.

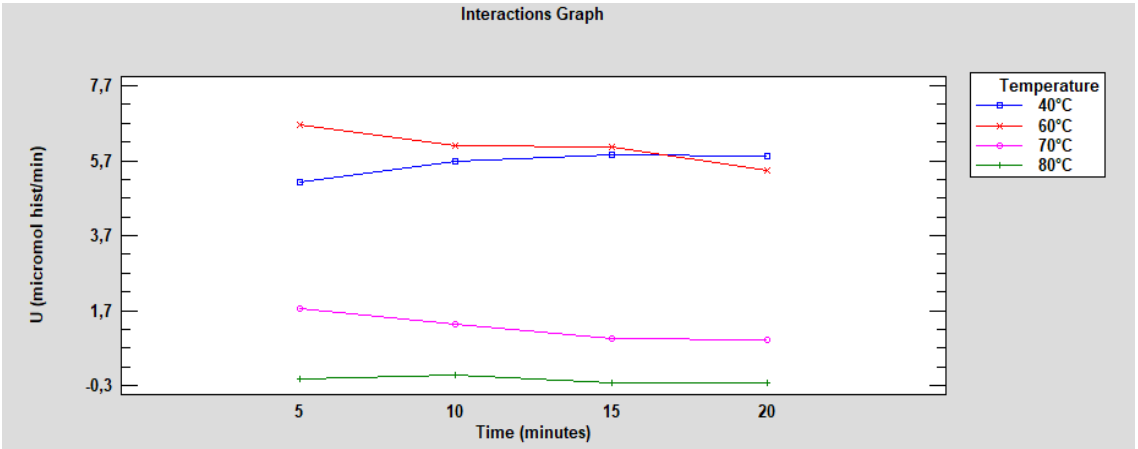

***Figure* 3-23** *Interaction graph.*

From the picture it is possible to observe that the 40°C line is characterized by a positive slope meaning that time increases the activity of the enzyme. All the other slopes appear to be negative, so if the enzyme is kept at the considered temperatures up to 20 minutes it loses its activity, confirming the previous results.

To conclude it is possible to state that an overall interaction is detected and that generally the considered factors, thermal treatment (or abuse for the 40°C) temperature and time, do not act independently: a change of one of the two may provoke a change into the effect of the other one.

The simple ANOVA test was carried between the control (untreated, time-zero) sample data and the families of samples, namely 40°C, 60°C, 70°C and 80°C separately. The goal of this study is to find the effect of the treatment at different temperatures on the DAO activity.

According to the results no significant difference ($p>0.05$) was found between the activities of the untreated enzyme and the 40°C treated one, while for the other temperatures tables and graphs follow.

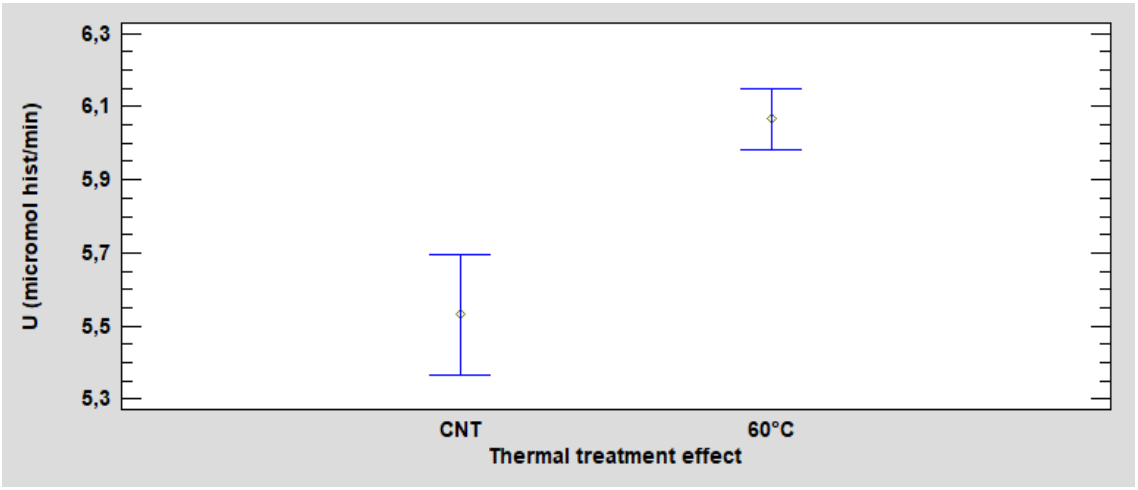

***Figure* 3-24** *one way ANOVA test-effect of the treatment at 60°C.*

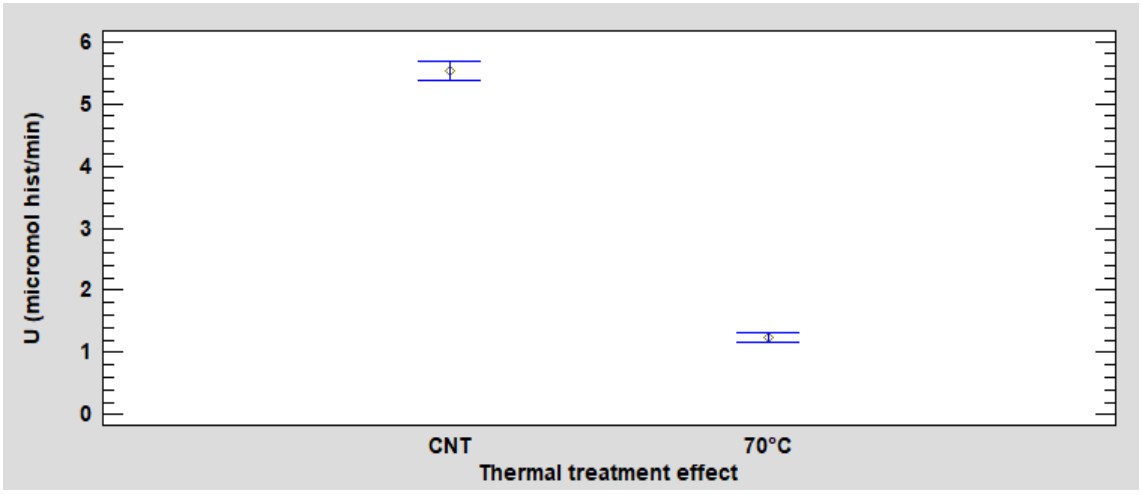

Figure 3-25 *one way ANOVA test-effect of the treatment at 70°C.*

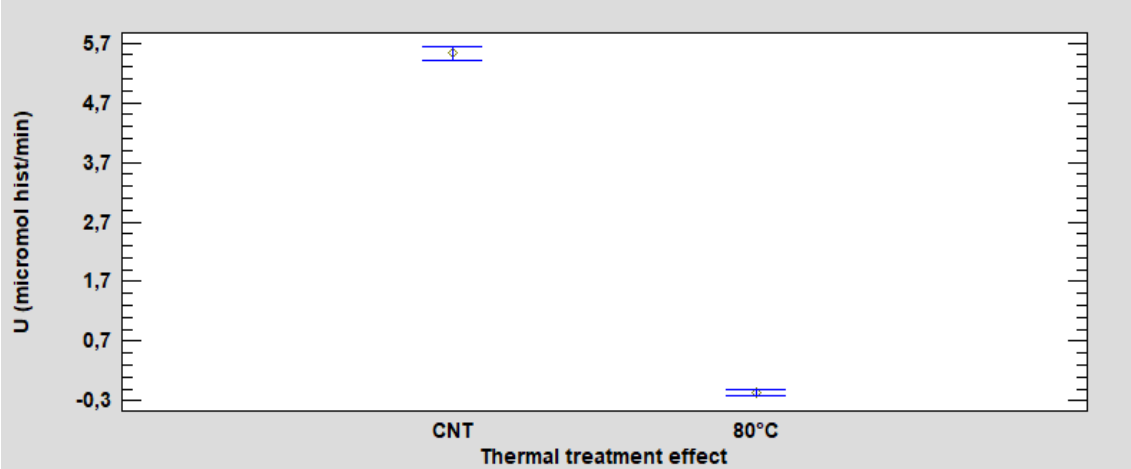

Figure 3-26 *one way ANOVA test-effect of the treatment at 80°C*

It can be observed that the thermal treatment has got a significant effect on DAO resistance in all the left cases, weakening it.

3.3 Conclusions, industrial applications and further studies

The analysis of results brings to the following conclusions:

- Resistance to pH: the enzyme is only active in neutral or basic environments and its activity increases during time, specifically up to 60 days of storage at 4°C.
- Storage conditions, neutral buffer: the enzyme maintains its original activity if stored either at -18°C or at room temperature (23±1°C) up to 7 days of time.
- Thermal treatments, neutral buffer: the enzyme loses its activity if the temperature reaches 80°C. If time increases up to 20 minutes the activity is enhanced at 40°C, while it gets worse at all the remaining temperature conditions.

Considering what has been found, in order to process and store foods enriched of DAO enzyme, the proper industrial operative conditions must be set to achieve the best enzyme preservation. Generally conditions of storage as 4°C, -18°C and room temperature conditions are adequate for the maintenance of the enzyme activity.

Regarding the thermal treatments for instance, considering the example of the fish processing industry or study field, some thermal-mild inactivation techniques could be applied respecting both the safety requirements and the enzyme conditions of activity. One of the heat treatments already applied to liquid foods, like milk and juices and studied on fish is the microwave one: in it resulted that such a heating up to 60°C (core temperature) brought to 4 log CFU/g reduction of *L. monocytogenes* in catfish [27]; such a condition would guarantee a good activity of the enzyme, and so histamine degradation, according to what was found in this work. Anyway, it

was also found that a treatment above 70°C would be necessary for a complete destruction of pathogens like *L. monocytogenes* and *Staphylococcus aureus* [27]: depending on the treatment time and reached temperature the enzyme could possibly maintain a certain activity, but further analysis would be appropriate. Another method of thermally mild treating foods is the sous vide cooking, in particular the most recent practice of the low-temperature sous vide cooking involving seafood: here temperatures are around 42-60°C, and a usual reheating is always suggested before the product consumption [27]. The process conditions of low-temperature sous vide cooking would belong to the acceptable range of temperature for the enzyme resistance, anyway if a further cooking is needed for an almost complete microbial growth prevention, then DAO could be deactivated. About non-thermal inactivation methods fish products are also processed with smoking techniques, particularly the cold smoking that reaches temperatures below 33°C: the smoking follows the salting and the drying - so eventually the evaluation of the enzyme resistance to these two practices would be needed - while a 4°C storage must be also provided [27]. Finally, among the mild inhibition methods belonging to the seafood processing, the superchilling techniques represents another possibility: called also partial freezing, this practice consists in the lowering of the operative temperature 1-2°C below the initial freezing point of the product [27]. About the pH, it was measured in tuna cans and it resulted having a value around 6, so not acid enough to degrade the proteinic structure of DAO enzyme.

Regarding further investigations in this field of study, starting from these results, it would be useful to assess the activity of the encapsulated form of porcine DAO enzyme, at the same conditions of pH and temperature. The final idea would be inoculating the DAO in histamine-rich foods inside capsules made of biodegradable and biocompatible substances, in order to let the enzyme be applicable in all the process conditions in which its free version would lose its activity. The protection would work as a proper shield, preventing the protein damage due to low pH values or high temperatures typical of pasteurization, sterilization or cooking practices. Other parallel studies could eventually regard the enzyme activity evaluation of the vegetal version of DAO, at the same considered conditions of pH and temperature, since it represents one the of the newest and most promising approach to the histamine intolerance disorder.

Appendix

pH treatment tables:

Table A.2 *1 hour of pH treatment.*

Samples	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	3.55	0.593	100.0	0
PIPES	4.02	0.460	114.3	10.32
pH 3	0.01	0.028	0.3	0.68
pH 5	0.71	0.164	19.8	2.27
pH 7	3.80	0.288	108.3	12.09
pH 9	3.78	0.423	107.8	14.46

Table A.3 *7 days of pH treatment.*

Samples	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	3.55	0.593	100.0	0
PIPES	4.41	0.692	124.6	1.50
pH 3	-0.01	0.119	0	3.70
pH 5	0.27	0.036	7.6	0.24
pH 7	4.65	0.660	131.6	5.24
pH 9	4.06	0.724	114.8	10.42

Table A.4 *60 days of pH treatment.*

Samples	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	3.55	0.593	100.0	0
PIPES	4.73	0.276	135.3	14.16
pH 3	-0.04	0.091	-1.0	2.31
pH 5	0.11	0.083	3.0	1.25
pH 7	4.85	0.114	139.2	13.62
pH 9	5.17	0.105	149.0	14.67

pH treatment tables – statistical analysis:

Table A.5 *ANOVA test-pH grouping.*

pH	Points	Average LS	Sigma LS	Groups
3	72	-0.01	0.052	A
5	72	0.36	0.052	B
9	72	4.34	0.052	C
7.2	72	4.39	0.052	C
7	72	4.43	0.052	C

Table A.6 *ANOVA test-time grouping.*

Time	Points	Average LS	Sigma LS	Groups
1 hour	120	2.46	0.040	A
7 days	120	2.68	0.040	B
2 months	120	2.96	0.040	C

Table A.7 *one way ANOVA test-effect of time on pH 7.2 sample.*

Time, pH 7.2	Points	Average	Groups
0	24	3.55	A
1 hour	24	4.02	B
7 days	24	4.41	BC
2 months	24	4.73	C

Storage conditions tables:

Table A.8 *1 hour of storage conditions treatment.*

Samples	U (µmol hist/min)	SD	Residual activity (%)	SD residual activity
CNT-t0	5.53	0.985	100.0	0
-19°C	5.47	0.541	99.9	11.73
Room T	5.69	0.541	103.8	8.95

Table A.9 *7 days of storage conditions treatment.*

Samples	U (µmol hist/min)	SD	Residual activity (%)	SD residual activity
CNT-t0	5.53	0.985	100.0	0
-19°C	4.93	0.371	91.6	21.55
Room T	4.54	1.385	86.5	38.61

Storage conditions tables – statistical analysis:

Table A.10 *ANOVA test-time grouping.*

Time	Points	Average LS	Sigma LS	Groups
7 days	48	4.73	0.112	A
1 hour	48	5.58	0.112	B

Thermal treatments tables:

Table A.11 *40°C thermal treatment.*

Sample	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	5.53	0.985	100.0	0
5 min	5.51	0.629	100.4	8.70
10 min	5.68	0.362	105.4	23.26
15 min	4.39	0.035	108.1	18.35
20 min	5.84	0.336	107.0	12.46

Table A.12 *60°C thermal treatment.*

Sample	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	5.53	0.985	100.0	0
5 min	6.66	0.093	123.0	21.92
10 min	6.09	0.136	112.0	16.37
15 min	6.08	0.066	112.0	17.53
20 min	5.44	0.165	100.1	14.85

Table A.13 *70°C thermal treatment.*

Sample	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT_t0	5.53	0.985	100.0	0
5 min	1.77	0.328	32.3	6.69
10 min	1.32	0.121	24.2	3.40
15 min	0.96	0.077	17.7	2.71
20 min	0.91	0.129	17.0	4.55

Table A.14 *80°C thermal treatment.*

Sample	U (µmol hist/min)	SD	Residual activity (%)	SD res. activity
CNT-t0	5.53	0.985	100.0	0
2 min	-0.04	0.136	-0.7	2.57
5 min	-0.14	0.094	-2.5	1.79
10 min	-0.03	0.106	-0.6	2.03
15 min	-0.23	0.032	-4.2	1.24
20 min	-0.24	0.020	-4.5	1.05

Thermal treatments tables – statistical analysis:

Table A.15 *ANOVA test-time grouping.*

Time (minutes)	Points	Average LS	Sigma LS	Groups
20	96	2.99	0.022	A
15	96	3.17	0.022	B
10	96	3.26	0.022	C
5	96	3.35	0.022	D

Table A.16 *ANOVA test-temperature grouping.*

Temperature	Points	Average LS	Sigma LS	Groups
80°C	96	-0.16	0.022	A
70°C	96	1.24	0.022	B
40°C	96	5.63	0.022	C
60°C	96	6.07	0.022	D

Table A.17 *one way ANOVA test-effect of the treatment at 60°C.*

Sample	Points	Average	Groups
Control	24	5.53	A
60°C	96	6.07	B

Table A.18 *one way ANOVA test-effect of the treatment at 70°C.*

Sample	Points	Average	Groups
70°C	96	1.24	A
Control	24	5.53	B

Table A.19 *one way ANOVA test- effect of the treatment at 80°C.*

Sample	Points	Average	Groups
80°C	96	-0.16	A
Control	24	5.53	B

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