UNI**CA**

UNIVERSITÀ
DEGLI STUDI
DI CAGLIARI

Ph.D. DEGREE IN
Molecular and Translational Medicine

Cycle XXXV

TITLE OF THE Ph.D. THESIS

Proteinaceous inhibitors of α-amylase and α-glucosidase from common bean (*Phaseolus vulgaris* L.): biochemical characterization and phylogenetic analysis of Sardinian cultivars

Scientific Disciplinary Sector

BIO/10

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Final exam. Academic Year 2021/2022
Thesis defense: April 2023 Session

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Abstract

The common bean *Phaseolus vulgaris* is a good source of protein fiber and bioactive compounds. Among these proteinaceous α-amylase inhibitors (α-AIs) and α-glucosidase inhibitor could play an important role in weight loss and control of glycemic index above all in overweight and diabetic subjects.

In this study 10 poorly studied Italian *P. vulgaris* cultivars were screened about their content in α-amylase/glucosidase inhibitors, antinutritional factor phytohemagglutinin (PHA), and about the expression of α-AI gene and their phylogenetic relationship.

All the cultivars presented α-glucosidase inhibitor activity, while α-AI was missing in two of them. Only Nieddone cultivar (ACC177) had no hemagglutination activity and its α-AI was extracted, purified and characterized.

The purified inhibitor has a tetrameric structure with a molecular weight of about 42 kDa, a temperature optimum of about 40°C and two pH optima (5 and 6.5).

Thermal stability is remarkably important, since the inhibitory activity was maintained at 25% after 5 hours of incubation at 100°C. The purified inhibitor had the highest affinity toward insect α-amylase about 3-fold more susceptible than human salivary amylase and porcine pancreatic α-amylase.

The kinetic characterization showed a mixed-type inhibition, suggesting a multiple site protein/protein interaction between enzyme (α-amylase) and inhibitor.

Molecular studies showed that α-AI is expressed in all cultivars and a close similarity between the two cultivars Pisu Grogu and Fasolu α-AI and isoform α-AI-4 emerged from the comparison of the partially reconstructed primary structures.

Moreover, from mechanistic models emerge the interaction network that connects the α-AI with the α-amylase enzyme characterized from two interaction hotspots (Asp38, and

Tyr186), giving some insights for the analysis of α-AI primary structure from the different cultivars, particularly about structure-activity relationship.

This study can be lay the basis for increase the knowledge about this class of proteins and developing commercial preparations from Italian common beans cultivars.

Sommario

Il fagiolo comune (*Phaseolus vulgaris*) è un'ottima fonte di fibre proteiche e di composti bioattivi. Tra questi, l'inibitore dell'α-amilasi (α-AI) e l'inibitore dell'α-glucosidasi svolgono un ruolo importante nella perdita di peso e nel controllo dell'indice glicemico, soprattutto in soggetti obesi e diabetici.

In questo lavoro sono state analizzate 10 cultivar italiane di *P. vulgaris* ancora poco studiate per quanto riguarda il loro contenuto di inibitore dell'α-amilasi/glucosidasi, di fattori antinutrizionali quali la fitoemoagglutinina (PHA), per l'espressione del gene α-AI e per le relazioni filogenetiche che intercorrono tra queste varietà di fagiolo.

Tutte le cultivar analizzate inibiscono l'enzima α-glucosidasi, mentre l'α-AI è assente in due varietà.

Solo la cultivar Nieddone (ACC177) non presenta attività emoagglutinante e per questo motivo si è deciso di estrarre purificare e caratterizzare α-AI a partire da questa varietà.

L'inibitore purificato presenta una struttura tetramerica con un peso molecolare di circa 42 kDa, un optimum di temperatura di circa 40°C e due optima di pH (5 e 6,5).

Risulta essere termicamente stabile dal momento che il 25% l'attività inibitoria si è preservata dopo 5 ore di incubazione a 100°C. È stato osservato che l'inibitore purificato possiede la massima affinità verso l'α-amilasi di insetto, circa 3 volte più sensibile dell'amilasi salivare umana e dell'α-amilasi pancreatica suina.

La caratterizzazione cinetica ha mostrato un'inibizione di tipo misto dell'α-amilasi, evidenziando la probabile presenza di più siti di interazione tra enzima e inibitore.

Dagli studi molecolari è emerso che l'α-AI è espressa in tutte le cultivar e che gli inibitori delle cultivar Pisu grogu e Fasolu risultano essere più simili l'isoforma α-AI-4 rispetto all'isoforma α-AI-1.

Inoltre, gli studi quanto-meccanici hanno messo in evidenza i siti di interazione tra α -AI e l'α-amilasi corrispondenti alle posizioni Asp38 e Tyr186. Questa informazione può contribuire all'analisi delle sequenze aminoacidi della proteina α -AI delle varie cultivar, dando informazioni sul rapporto struttura-attività.

Questo studio può nel suo insieme costituire la base per aumentare le conoscenze su questa classe di proteine e per sviluppare integratori alimentari a partire da varietà di fagiolo italiano.

1. State of the art

1.1 Introduction

Overweight and obesity are one of the major health concerns of our times, being considered the fifth leading risk for global deaths worldwide [1]. According to WHO, more than 1.9 billion adults were overweight in 2016, and over 650 million of these were obese.

It is estimated that globally there are more people obese than underweight in every region, except for some parts of sub-Saharan Africa and Asia.

The imbalance between energy expenditure and diet calorie intake has been identified as the cause of this condition, being mainly caused by the decrease in physical activity and the shift towards diets rich in sugars and fats, and low in fibers and micronutrients (vitamins, minerals and so on) [2].

Besides, overweight and obesity are related to many common health consequences, increasing risk for cardiovascular diseases, hypertension [3], dyslipidemia, sleep apnea, knee osteoarthritis [4], and certain types of cancer [5].

Among the main comorbidities, type 2 diabetes requires special attention.

The close relationship between obesity and diabetes led to the coining of the term 'diabesity'[6,7] a concept retaken in a very recent study that shown as obesity and unhealthy lifestyle influence the onset of type 2 diabetes regardless of genetic predisposition [8].

A progressive defect in insulin secretion associated with a progressive rise in insulin resistance led to the passage from obesity to diabetes [9]. More recently, obesity has even been identified as a leading risk factor for hospitalization and poor clinical outcome of SARS-CoV2 patients during COVID-19 pandemic [10].

Several dietary strategies have been suggested to tackle these issues, affecting carbohydrate and lipid metabolism. The most straightforward solution could be to reduce

the carbohydrate portions or their absorption introducing dietary fibers [11]. However, most people distaste such dietary modifications and report gastrointestinal issues [12]. Similar results can be obtained by inserting in the diet starches that resist digestion in the small intestine, thus acting like dietary fibers [2]. These starches are naturally found in several seeds, legumes and unprocessed whole grains, with a low glycemic index (GI).

A different approach could involve the use of some phytochemicals to slow down carbohydrate absorption. This could be achieved by preventing their intestinal hydrolysis, inhibiting the necessary enzymatic activity, namely amylase and glucosidase [13,14].

α–Amylase (α–1,4–glucan–4–glucanohydrolases, E.C. 3.2.1.1) is a glycoprotein catalyzing the endohydrolysis of (1→4)-α-D-glycosidic linkages in polysaccharides containing three or more (1→4)-α-linked D-glucose units [15]. During the reactions catalyzed by mammalian and bacteria α -amylases, as shown in Figure 1.1, the formation of covalent β-linked carbohydrate–enzyme intermediates in which are involved two aspartate residues and one residue of glutamate occurs [16,17].
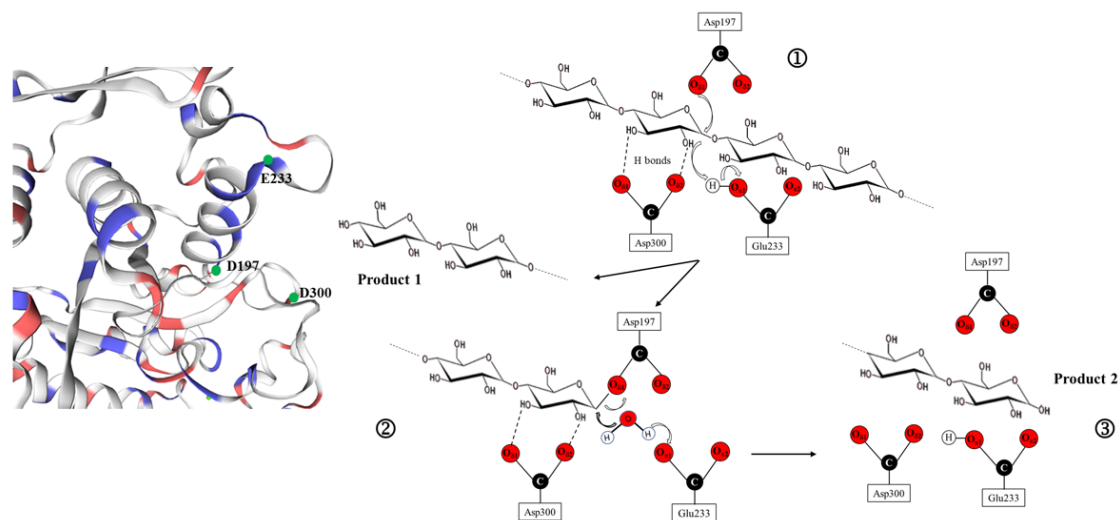
Figure 1.1 Reaction mechanism of PPA. 1) Nucleophilic attack of carboxylate group of Asp197 onto C1 of the α-(1-4) glycosidic linkage and release of the first product; 2) hydrolysis of the α-carboxylacetal covalent intermediate; 3) Release of the second product in α-anomeric configuration.

The human isoform is a single peptide chain of 512 amino acids (about 57 kDa), requiring calcium ions for structural integrity [18,19].

This is one of the major secretory enzymes of the salivary glands and pancreas, playing a central role in the digestion of starch and the absorption of deriving simple sugars.

The cleavage of α-D-(1-4) glycosidic bonds release shorter oligosaccharides retaining the α-anomeric configuration, called dextrins (a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units with both α-1,4 and α-1,6 linkages). Other intestinal brush border enzymes (namely α-glucosidase, maltase and isomaltase) in turn further hydrolyze dextrins, and the resulting sugars can then be absorbed. No terminal glucose or α-D-(1-6) glycosidic bonds are affected by amylase activity [20]. Structurally, all α-amylases are usually folded into three domains: A, B and C. Domain A consists of a $(\beta/\alpha)_8$-barrel, which is the core structure of the enzyme. Domain B is a sheet of four anti-parallel β-strands with a pair of anti-parallel β-strands. The binding site for calcium

ion is located in this domain. Domain C consists of eight β-strands forming a Greek key motif.

Thus, α–amylase represents the crucial step in the initiation of the process of digestion and absorption of dietary polysaccharides [21]. Therefore it can be the target for specific enzyme inhibitors, allowing to reduce the rate of carbohydrate absorption, the real energetic intake after a meal, and the subsequent glycemic peak [20,22].

Several alternatives have been proposed: acarbose (commercially known as Prandase®, Glucobay®, Precose®) is the best known α-amylase inhibitor. This is a low MW synthetic tetrasaccharide analogue (Figure 2), used as prescription drug to tackle hyperglycemia in treatment of type 2 diabetes mellitus [2]. Miglitol and voglibose are other drugs belonging to the same class. As shown by several clinical studies, acarbose effectively improves insulin sensitivity in subjects with impaired glucose tolerance or type-2 diabetes, reducing cardiovascular risks in subjects with metabolic syndrome (a cluster of risk factors including high triglycerides, low high-density lipoprotein cholesterol and hypertension) [2,23].

(a) Acarbose

(b) (-)-Epicatechin

(c) Chlorogenic acid

(d) Oleanolic acid

(e) Ursolic acid
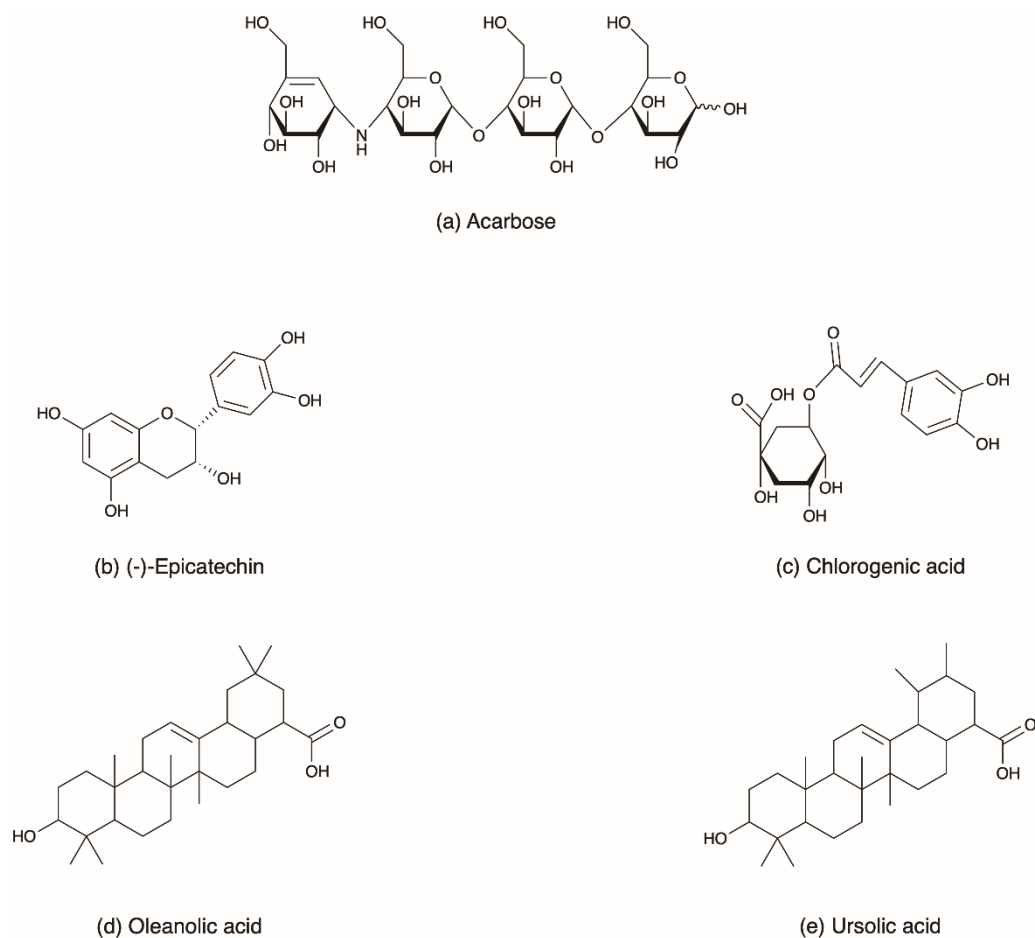
Figure 1.2 some of the described low MW α-amylase inhibitors.

Several α-amylase inhibitors are also naturally present in plants, where they act as defense chemicals from biotic stress [2,24]. Over 800 plant species have been reported to have antidiabetic activity [20]. In particular, alkaloids, polysaccharides, steroids, glycopeptides, terpenoids, and several polyphenols (including hydroxycinnamic acids, chalcones, epicatechin, flavans, and anthocyanins, as shown in Figure 1.2) have been described as possible low MW drug candidates [14,25–27].

Among natural inhibitors, however, much attention has been paid to certain proteins which interact with α-amylase, thus providing a protein-protein interaction capable of reducing hydrolyzing activity. Proteinaceous α-amylase inhibitors can derive from

microbial and plants sources [28]. The latter can be further divided into six different classes: the knottin-like type, the Kunitz type, the thaumatin-like type, the γ-thionin-like type, the cereal type, and the lectin-like type (recently well reviewed in [22]). The first three classes only affect the amylases insect and can be therefore used in agriculture as biological pest control methods.

However, some classes can interact with mammalian (and human) enzymes and thus represent a potential drug candidate for antidiabetic and overweight treatments. The greatest interest has been addressed towards legume lectin-like glycoproteins extracted from several legumes [29,30]. In particular, the commercial proprietary Phase2® is produced from the common bean *Phaseolus vulgaris*, as well as other similar "carb controller" products [2,12,31].

1.2 Amylase inhibitors found in *P. vulgaris*

Among the described α-amylase inhibitors, the proteinaceous ones from *P. vulgaris* L. (α-AIs) show a particularly high potential [32]. In fact, the common bean is a well-widespread cultivation worldwide, with several positive nutritional features. Besides, these inhibitors are generally recognized as safe (no side effects found, as for other plant inhibitors [33]).

α-AIs are lectins (carbohydrate-binding proteins, causing agglutination or precipitation of cells, glycoconjugates, including glycoproteins). *P. vulgaris* expresses three main classes of lectins: α-AIs, phytohemoagglutinins (PHA), and arcelins (ARL), as protection agents of seeds from biotic (i.e. insect predation) and abiotic stress (i.e. drought, salinity and wounding) [34,35], all deriving from a single ancestral gene [36]. PHA is a mitogen, affecting the intestinal mucosa of mammals, whereas ARL prevent absorption of nutrients

in insect larvae [37]. These components are regarded as antinutritional factors [38], but thermal denaturation (typically reached during cooking) inactivates anti-nutrient agents, adding nutritional value due to their high sulfur aminoacid content [35].

Three main α-AIs can be found in common beans: isoform 1 (α-AI1), isoform 2 (α-AI2), and α-amylase inhibitor-like (α-AIL) [38,39]. All these proteins are specifically able to inhibit animal α-amylases, not affecting plant amylases [34]. Due to their activity against insect amylases, these proteins have been proposed as insecticides against bruchid pests [40]. In fact, their genes have been also transgenically expressed in other plants (such as tobacco and peas) to confer resistance to various insect pests [41,42].

On the other hand, only α-AI1 has a specific effect on mammalian (and thus human) amylases, being therefore the target for nutraceuticals clinical studies. Heterologous expression of α-AI1 in yeasts has also been recently described to improve potential large scale production [43].

1.2.1 Amylase inhibitors genes

As aforementioned, seven types of natural protein inhibitors of α-amylases (α-AIs) have been identified, and six of these, namely the knottin-like type [44], the γ-thionin-like type [45], the cereal type, the Kunitz type, the thaumatin-like type and the lectin-like type [46], are extracted from plants. The other type has been isolated from bacteria [47]. The molecular weights of these inhibitors range from 3 kDa to 23 kDa [44]. The knottin-like type, the Kunitz-type and the thaumatin-like type are able to inhibit amylase only in insects [48].

The α-AI lectin-like glycoproteins have been isolated from legumes. They were named for their high degree of amino acid sequence homology compared to the lectin proteins abundantly expressed in these plants.

The currently known α-AI isoforms present in the different cultivars of the legume, *P. vulgaris*, are α-AI1 [49,50], α-AI2 [51], α-AIL [52], α-AI4 and α-AI5 [53]. All the α-AI isoforms are encoded by genes that are part of a gene cluster, which includes *dlec1* (encoding lectin phytohemagglutin PHA-E), *dlec2* (encoding leukophytoagglutinin PHA-L) and *Arc* (encoding arcelin) [54,55].

According to evolutionary studies, the gene cluster may have originated following gene duplication events [54]. The gene cluster encodes proteins that share similar characteristics in their secondary and tertiary structures, as they are basically made up of antiparallel β-sheets. ARL differ from PHA-E and PHA-L through the absence of a short amino acid sequence (gap deletion G3), which, if present, forms a short loop. α-AI1 and α-AI2 differ from ARL through the absence of two sequences (gaps deletions G1 and G2), which, if present, form two other loops.

Ultimately, the proteins α-AI1 and α-AI2 have the shortest sequences due to the deletion gaps G1, G2 and G3. The α-AIL protein (α-amylase inhibitor-like protein previously called α-AI3) differs from α-AI1 and α-AI2 mainly due to the insertion of 15 amino acids around position 115 (G2) of the primary structure [52].

The α-AIs in the bean, similar to the other proteins encoded by the gene cluster, are synthesized in the endoplasmic reticulum (ER) as inactive preproteins to become active proteins once transported to the vacuoles. The transition from inactive to active protein involves several post-translational modifications by which the precursor is *N*-glycosylated and cut (except in the case of the α-AIL isoform) at a conserved Asn residue

to give rise to two subunits (called α and β) that assemble non-covalently (Figure 1.3) [34,50,56–58].
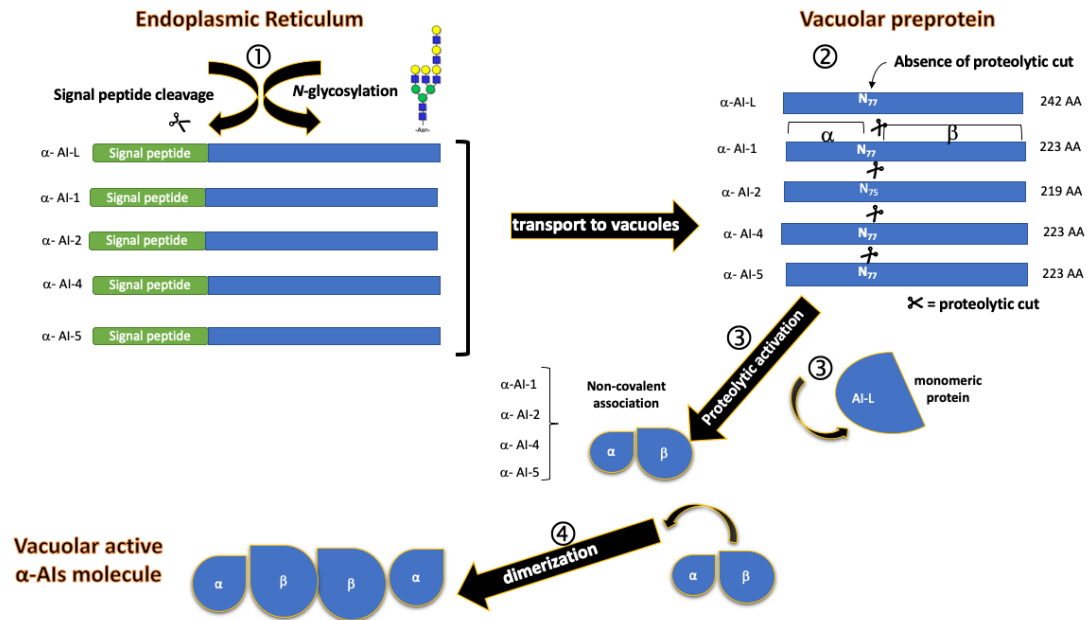
Figure 1.3 Different α-AIs isoforms present different susceptibility to proteolytic activation.

The α-AIs of *P. vulgaris* generally show only small differences in their primary structures and in their *N*-glycosylation sites. αAI-1 was the first isoform of *P. vulgaris* to be isolated in 1945 [59], molecularly characterized [34,60] and crystallized [61]. In the ER, the preprotein undergoes cleavage of the signal peptide and *N*-glycosylated sites on residues of Asn12, Asn65 and Asn140 (the numbers refer to the positions of the residues in the mature protein). Through the secretory pathway, the protein is transported to the vacuoles, where it undergoes a second proteolytic cut. Specifically, vacuolar proteases cut at the conserved Asn77 residue (the number indicates the position of the residue after removal of the signal peptide) and also at the Asn223 to remove the C-terminal portion, which is then used to direct the peptide transport to the vacuoles. These post-translational modifications give rise to the α and β subunits, with molecular weights of 7,800 Da (77 amino acid residues) and 14,600 Da (146 amino acid residues), respectively [58,62]. As

shown in Figure 1.4, in the native protein, the protein subunits are then organized in a tetrameric structure $\alpha_2\beta_2$ [53,55].
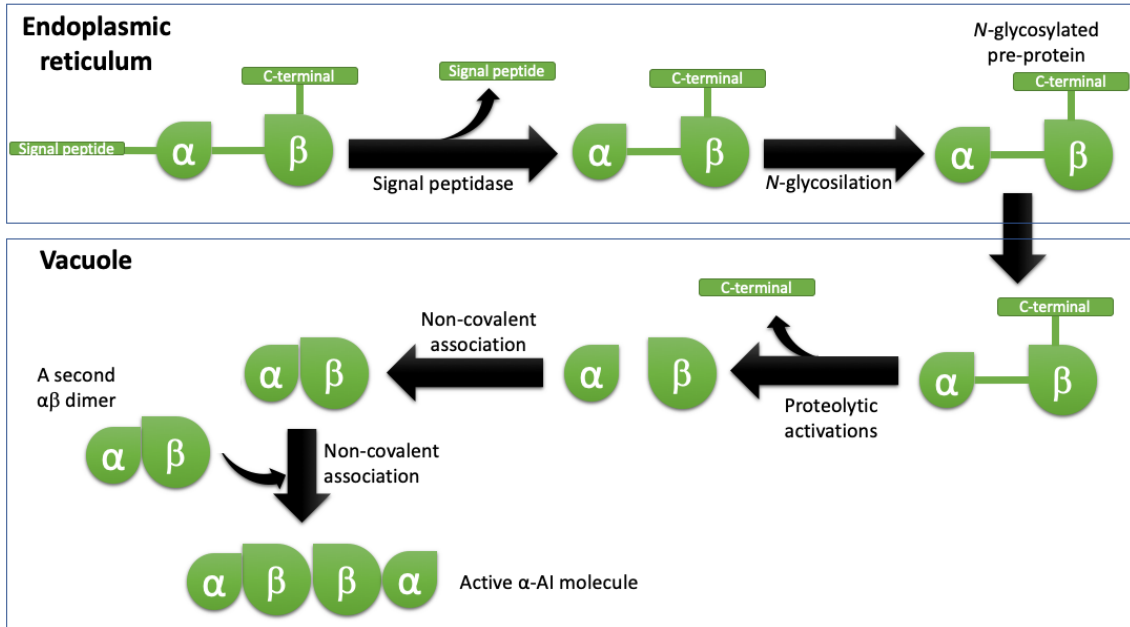
Figure 1.4 Post-translational modifications of α-AI1, α-AI2, α-AI4, and α-AI5. Proteolytic activation includes the removal of signal peptide, and the proteolytic scission of subunits α and β. Then, dimerization leads to αβ dimers, that associate in pairs to form the heterotetramer α2β2, the active α-AI molecule (adapted from (Lee et al., 2002)).

Site-specific mutagenesis experiments have demonstrated the importance of the amino acid residue in position 77 of the mature protein for the acquisition of the native structure. In fact, the removal of the proteolytic cleavage site at Asn77 by substitution of the ATT codon with a GAT codon (Asn77→Asp77) in the gene α-AI1 encoding the α-AI1 isoform, leads to the expression of an inactive inhibitor [56]. In the αAI-2 isoform, the proteolytic cleavage that gives rise to the α and β chains, occurs at Asn75. This isoform has an α subunit that is shorter, by two amino acids, than the α subunit of α-AI1 (Ser56 and Tyr57 are missing). It is interesting to note that α-AIL, while retaining Asn77 in its primary structure, does not undergo proteolytic cleavage, so it is a monomeric protein [52].

By exploiting the ability of the protein isoform, α-AI1, to interact with mammalian α-amylases, Bompard-Gilles and coworkers crystallized the α-AI1/PPA complex (porcine pancreatic α-amylase-α-AI1) in order to define the interaction domains that are established between the two proteins [61]. Contrary to the results obtained in previous studies [63], the analysis of the crystals showed that α-AI1 consists of two dimers, and each is able to interact with a pancreatic enzyme molecule, PPA. Its secondary structure is devoid of α-helices and contains an abundance of β-antiparallel sheet. In the inhibition process promoted by αAI-1, two hairpin loops in the inhibitor (L1 and L2) play an important role. The amino acid residues in the loop make direct and indirect hydrogen bonds via H_2O molecules with the enzyme (see below). L1 includes residues 29–46, and L2 includes residues 171–190. The inhibitor-enzyme interactions lead to conformational modifications of α-AI1 projecting the Tyr37 and Asp38 residues of L1 and the Tyr186 and Tyr190 residues of L2 into the active site of the enzyme, where three amino acid residues directly involved in acid-base catalysis events are present (Asp197, Glu233 and Asp300, as shown in Figure 1.5). Tyr37 of the inhibitor, as a result of conformational changes induced by interactions with the enzyme, forms hydrogen bonds with the Glu233 residue and part of the Asp300 residue present in the active site of the enzyme. The latter is also engaged in binding with a chloride ion. The flanking Asp38 residue of the inhibitor assumes a position favorable for the formation of a hydrogen bond with His201, which is also present in the active site of the enzyme. The formation of this hydrogen bond facilitates the interaction of Tyr186 with the enzyme, which is projected into the heart of the catalytic site, forming hydrogen bonds with the catalytic nucleophile, Asp197 [64]. The orientation of the L2 loop allows the formation of a hydrogen bond with the Asp197 residue of the enzyme, which will not be able to participate in the catalytic event as a

nucleophile [64]. Asp300 in the enzyme, the third residue of the catalytic triad involved in the acid-base catalysis with the substrate, is blocked, as it forms two hydrogen bonds with the inhibitor: the first bond is established between the carboxyl of its side chain and the hydroxyl group of Ser189 in the inhibitor, and the second bond is established between its carbonyl oxygen and Tyr190 in the inhibitor.
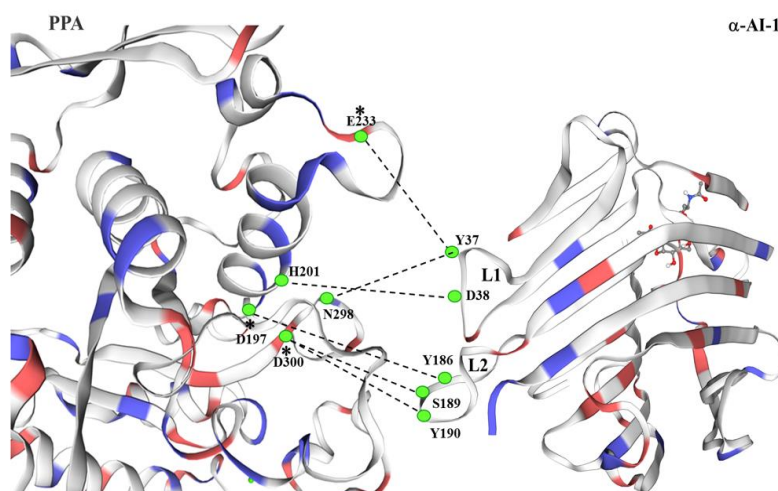
Figure 4

*Figure 1.5 Three-dimensional model structure of the PPA/α-AI1 complex. One monomer unit of α-AI1 (template 1dhk.1.B) in interaction with a molecule of PPA (template 3l2l.1.A) is shown. The two hairpin loops (L1 and L2) of α-AI1 interacting directly with the active site of PPA are highlighted. The α-AI1 tyrosine, aspartate and serine sidechains (Y37, Y186, Y190, D38, S189), important to the interaction with catalytically important residues of PPA enzyme (E233, D197, D300, H201, N298) are reported with the one-letter code. The * indicate the three amino acids of α-AI1 directly involved in the catalytic mechanism. The 3D structures were generated using SWISS-MODEL (http://swissmodel.expasy.org) server.*

Additional molecular information on *P. vulgaris* α-AIs was obtained after the isolation of the genes encoding the protein isoforms α-AI2 and α-AIL [51,52,55]. The reconstruction of the primary structure of the two proteins allowed to define their hypothetical three-dimensional structures using the crystal structure obtained by Bompard-Gilles and colleagues for the PPA/α-AI1 complex as a model [61]. Despite the high degree of

similarity of the three isoforms, α-AI1, α-AI2 and α-AIL, their specificities to different α-amylase enzymes vary. While α-AI-1 inhibits the activity of pig α-amylases and those of the insects *T. molitor*, *C. maculatus* and *C. chinensis*, the other two isoforms do not act on these enzymes. α-AI2 inhibits the α-amylase of the *Z. subfasciatus* beetle (ZSA), while α-AI1 does not. To explain whether these differences could be justified at the molecular level, the two isoforms, α-AI2 and α-AIL, were modeled in three dimensions using PPA/α-AI1 and PHA-L crystals as models [65]. Although all the isoforms had very similar three-dimensional structures, both α-AI2 and α-AIL differed from α-AI1 by some more or less extensive structural changes.

The interaction between α-AI1 and amylases seems to happen in the same site of acarbose [66,67].

The three-dimensional structures of these two isoforms, based on the information obtained from the crystallographic studies of the α-AI1/PPA complex, highlight the presence of the three residues (Arg74, Trp188, Tyr190) important for the inhibition of α-amylase. In particular, it has been suggested that the active inhibitor site consists of Trp188 (β subunit), Arg74 (α subunit) and Tyr190 (β subunit), resembling the Trp-Arg-Tyr motif of bacterial α-amylase inhibitor Tendamistat [68]. In this perspective, the proteolytic activation at Asn77 may be necessary to bring these residues in close proximity. Besides, two tyrosine residues (Tyr37 and Tyr186) from the two hairpin α-AI1 loops interact with two key residues in the enzyme active Asp197 and Glu233 [67]. These two nucleophilic and acid functions act as crucial catalysts in the amylase action. Besides, further protein-protein interactions involve areas away from the active site, including the loop 303–312, the loop at position 237–240, the loop 347–357, and the loop 140–150 from domain B [67]. However, some differences in α-AI1 inhibition have been

observed between human and pig pancreatic amylases, being possibly due to the aminoacidic sequence dissimilarity in the two enzymes [69]. For instance, some additional hydrogen bonds between inhibitor and human enzyme domain B have been observed, as well as different patterns of interactions in the loop regions 303–312 and 347–357 of domain A [67].

On the whole, this complex network of protein-protein interactions mimics the substrate binding in several subsites of the enzyme structure, inducing a steric hindrance process blocking the access of starch to the active site, which prevents hydrolysis. However, this process seems to be different if compared with the action of carbohydrate inhibitor acarbose. In this case, conformational changes have been reported in the flexible loop (residues 303–309), in the loop 237–240, and in the segment at position 140–150 of the domain B [66,67]. The main residues involved are the catalytic residue Asp300 and residue His305, which undergo deep conformational changes. After substrate binding, the flexible loop moves toward the inhibitor, leading to a reduction of the cleft breadth. On the contrary, when α-AI1 binds the enzyme, a tight-binding process occurs, pushing away the same loop toward the solvent. The loop region 351–359 in domain A undergoes significant structural modifications, while Asp 300 does not modify the orientation if compared with free amylase.

However, the process of α-AI1 has not been completely elucidated, being the role of some residues still not completely and universally understood [38]. This should encourage further studies, in perspective of the development of more (semi)synthetic inhibitors (see below).

All the α-AI isoforms, despite having a high sequence homology overall, differ consistently in their upstream and downstream regions, which contain the amino acid

residues responsible for the formation of hydrogen bonds with the active site of α-amylases [53]. It is probable that the low sequence homology in this region is responsible for each isoform's specificity for α-amylases. In fact, studies on the α-AI1 and α-AI2 isoforms have shown that the differences in these regions lead to a different orientation of the L1 and L2 hairpin loops when the inhibitor comes into contact with the active site of the enzyme. Knowing the structural details of this family of plant α-amylase inhibitors and their mechanism of action could help to understand their important roles in the control of endogenous amylases and in the protection against pathogens and parasites. Such pieces of information could also enhance the development of new inhibitors resembling the mechanism of α-AIs.

1.2.2 Structural properties

In the common bean, α-AI is a 43 kDa-residue polypeptide with three disulfide bridges that includes different isoforms (see above) [50,70]. As stated above, the active sites of amylases usually comprise three key amino acids, namely Asp197, Glu233 and Asp300 [71]. The α-amylase binding site could hold at least six monosaccharide structures and be cleaved between the third and fourth pyranose residues by a double displacement mechanism [48].

The inhibitor α-AI1 is purified from a variety of kidney bean seeds and is a 43 kDa tetrameric glycoprotein ($\alpha_2\beta_2$) with a Stokes radius of 29.0 Å, which corresponds to globular proteins. Chemical and enzymatic deglycosylation methods gave M_r calculated from SDS–PAGE experiments of about 7.8 and 14 kDa for α and β subunits, respectively [58]. All these protein fractions react with biotinylated ConA, thus indicating they are

glycosylated. The branched glycans linked to Asn65 and Asn140 of α-AI1 residues, protrude in the solvent at the back face of the dimer [37].

α-AI1 has in turn two isoforms in *P. vulgaris* cv Tendergreen, α-AI1 and α-AI1', which differ from each other by their isoelectric points and neutral sugar contents [50,72]. Both α-AI1 and α-AI1' give a single protein band when analyzed by PAGE in native conditions, with an estimated M_r of 43.6 and 39.8 kDa, respectively [50]. The yield of neutral sugar contents of α-AI1 and α-AI1' is 16% and 14% (*w/w*) respectively as estimated according to the phenol–H_2SO_4 method [73]. According to the vapor phase chromatography, the oligosaccharide chains of both inhibitors are mainly composed of mannose associated to a trace of xylose [74]. α-AI1 is a lectin-like amylase non-competitive inhibitor that has been found both in mammalian and two kinds of insects' amylases that come from *C. maculatus* and *C. chinensis* [22].

The occurrence of two extra-loops on the front face of α-AIs prevents these proteins from entering the substrate cleft of PPA; thus, the removal of these loops appears as a structural requirement for the α-amylase inhibitory activity of α-AIs. In addition, as discussed above (Figure 4), the proteolytic processing occurring at Asn77 residue, which cleaves the polypeptide chain of α-AI1 in an α-chain (residues 1–77) and in a β-chain (residues 78–223), is necessary to transform an inactive precursor in a fully active inhibitor [56].

Several studies confirmed that α-AI1 interacts with a large region that surrounds the substrate binding site of PPA in a slow process and forms a 1:2 stoichiometric complex which exhibited an optimum pH of 4.5 at 30°C [50,75]. The total buried area contributed by both molecules in this complex is about 3050 $Å^2$, which is reported to be one of the largest values for a protein-protein complex [61] [16]. α-AI1 was also similarly shown to interact with the *Tenebrio molitor* α-amylase [76].

1.2.3 Operational stability

Several factors affect α-AI activity, including pH, temperature and several ions.

While mammalian amylase presents the highest activity close to neutrality (6.5-7), the optimum of pH for inhibitory activity has been identified in a slightly acidic environment, in the range 4.5-5.5 [63,77–79], being almost inactive outside this range [50]. Lajolo and Finhardi Filho highlighted a specific difference between salivary (4.5) and pancreatic amylases (5.5) [79]. Other authors showed an optimum at neutrality using porcine pancreatic amylase, but no incubation was performed in this case [80]. Similar values of pH (5.25) have been also shown to positively affect inhibitor purification [81].

In fact, incubation time seems to greatly affect inhibitory activity, possibly explaining the different pH optima observed. A period of time comprised between 10' and 120' has been used to reach optimum activity [50,63,77]: shorter incubation was in fact necessary when operating at pH 4.5. These findings suggest that, to obtain *in vivo* effects, nutraceutic preparations based on α-AI should be ingested before the meals, or at least at the same time [38]. The pre-incubation pH could also enhance α-AI effects. For instance, 20' at pH 4 led to a boost of inhibition [36], even though these data were collected on insect amylases.

Incubation times and pH seem to interfere also when the temperature effect on α-AI is evaluated. In fact, when operating at pH 4.5, a maximum of activity between 22 and 37°C was observed, with no significant differences within this range [50]. Even Lajolo and coworkers showed only a slight increase in activity in the range 25-45°C, incubating inhibitor and enzymes at pH 5.4 [79]. On the contrary, a 10-fold increase in activity has been detected pre-incubating the inhibitor at amylase optimum pH (6.9) [63]. These

findings could be explained by the lower energy barrier at acidic pHs [79]. All studies agree on the complete loss of activity boiling at 100°C and refrigerating at 0°C [50,63,82]. In a study on transgenic peas, however, α-AI retained almost all the inhibitory effect when treated for 5' at 80°C (an almost complete drop in activity was observed for higher temperatures) [83].

Several ions also affect the binding between α-AI and amylase, and the consequent inhibitory effect. Particularly, calcium ion (5 mM) is important for the rate of initial binding [80]. Chloride ion is also necessary to reach optimum activity [79,80]. In fact, both these ions are usually present in the typical protocol commonly used for the determination of inhibitory activity. Potassium, magnesium and sulfate ions did not interfere with α-AI biological activity [80], whereas a positive effect of nitrate, bromide, iodide, and thiocyanate ions has been described [79].

Taken together, all these data show several discrepancies about the optimal operational conditions for inhibition activity. This suggests the need of further studies to evaluate more carefully the interactions between pH, temperature, and incubation times, and to identify more precisely the best conditions for processing and administration of possible α-AI-based preparations.

1.3 Clinical trials

According to WHO obesity is defined as abnormal or excessive fat accumulation. The body mass index (BMI), calculated by dividing a person's weight in kg by the square of height in meters (kg/m^2), represents a measure of obesity: a person is considered obese with a BMI $\geq$ 30.

The cause of excess body weight is an imbalance between energy intake and expenditure.

The WHO identifies two main factors which can cause the obesity condition: on one side the increasing intake of food rich in fat and sugar but lacking in vitamins and minerals, and on the other the decrease in physical activity due to an increase in sedentary works, urbanization and motorized transports [84].

Instead, diabetes is defined as a chronic, metabolic disease characterized by elevated levels of blood glucose leading to other health problems that mainly concern heart, blood vessels, eyes, kidneys and nerves (WHO, 2022). Type 2 diabetes is the most common form of diabetes mellitus and it is characterized by hyperglycemia, insulin resistance, and relative insulin deficiency [85].

It is considered that the aetiology of type 2 diabetes is strongly-diet related because of about 90% of diabetic patients are obese or overweight [86].

The spread of these health conditions and the necessity to implement the green economy led to the study of innovative drug, in particular from plant-based bioactive compounds, for their treatment. As mentioned above, many *in vivo* studies have shown that extracts of *P. vulgaris* have anti-obesity effects in animals and human models, presenting several advantages if compared to traditional dietary or pharmacological interventions [32,33]. On the contrary *in vitro* models have not been usually employed. In fact, the overall effect on blood glucose of α-amylase:α-AI interaction can be difficulty observed by such models. So, in this Review we focused only on *in vivo* studies.

1.3.1 *In vivo* studies on animal models

Many studies related to the administration of α-amylase inhibitor extracts to rats have been collected in a paper review by Carai and coworkers [87]. Unfortunately, the different

ways used to express enzyme and/or inhibitor activities in units rather than in absolute weight terms, make comparisons not easy to obtain.

The inhibition of the growth rate in rats fed with raw *P. vulgaris* beans and extract, changes biochemical parameters and alteration in organ histology, as reported since the 1960s [88–90]. Only a transitory pancreatic enlargement was observed in young healthy rats consuming raw kidney beans chronically, accompanied by a significant reduction in body content of lipid and a lower body weight gain compared to control group [91].

A weight loss was also observed after the administration of purified α-AI for 10 days, but it was accompanied by intestinal blockage (cecum level) by solidified digesta in healthy Lister Hodded rats, since starch digestion was almost completely blocked [92]. This effect, recorded only when the highest inhibitor concentrations (20 and 40 mg/kg) were administered, was linked to bacterial fermentation causing the hypertrophic growth of the tissue leading in some cases to the organ rupture.

On the contrary, using a different dry *P. vulgaris* extract, the administration of various doses (0, 50, 200 and 500 mg/kg) for 10 days did not imply side effects, but a reduction in rat body weight associated with a decrease in food intake was observed [93].

This anorexigenic effect, observed by many authors [94–97], may be related to the lectin activity on the intestinal brush border, by altering the release of cholecystokinin and glucagon-like peptides that play an important role in the control of appetite. This phenomenon has been observed also when high palatable food was administrated to rats [87,93].

It has been demonstrated that *P. vulgaris* extract is also effective in reducing glycemia in healthy and diabetic rats [96,97], as well as glucose absorption.

It is interesting that the antihyperglycemic activity is more effective if the *P. vulgaris* seeds are mixed with glibenclamide, an oral hypoglycemic agent. It was demonstrated that the combination of 300 g/kg (weight of seeds per body weight) with 0.20 g/kg body weight of gilbenclamide results in a safer and potent hypoglycemic and antihyperglycemic activity in chronic diabetic conditions [98].

Different results were obtained when crude white bean flour was administrated (1 g/kg body weight) to diabetic induced Wistar rats for 21 days; no significant decrease in blood glucose was recorded in this case [99].

These different results could be linked to the different degree of purification of the extract in accordance with what observed in human model (see below) to various dosages and to the absence of a joint action with hypoglycemic agents.

Similarly, no significant weight loss, change in organ weight, food consumption and blood biochemical parameters were recorded also in healthy Sprague-Dawley rats, that received white kidney bean extract at doses 4, 2 and 1 g/kg for 90 days [100].

Qin et *al.* suggest that this difference could be related to the experimental group state of health [100]. Healthy rats are in fact probably more resistant to α-AI effect compared to obese rats. Previous studies showed that amylase activity is higher in healthy subjects than in obese subjects (both rats and humans) [101,102], and so the inhibitor could act more effectively on healthy individuals.

The studies on animal models (as summarized in Table 1.1) have shown the potential of *P. vulgaris* extract as anorexigenic and antihyperglycemic agent although these properties depend on the type of extract administered, the duration of the trial and the health state of animals.

Ref.	Administration	Subjects	Side effects	Main results
[91]	Raw beans	Healthy rats	Transitory pancreatic enlargement	Reduction in body lipid level
[92]	Partially purified extract	Healthy rats	Intestinal blockage (cecum level) by solidified digesta	Weight loss
[93]	Dry extract	Healthy rats	No side effects	Reduction in rat body weight, glycemia and a decrease in food intake
[97]	Purified extract	Healthy and diabetic rats	No side effects	Reduction in glycemia and decrease in food intake in both group
[94]	Partially purified extract	Healthy rats	Not available	Reduction in normal and palatable food intake ang glycemia
[96]	Purified extract	Healthy rats	No side effects	Anoressigenic effect, reduction in weight gain, and blood glucose level
[98]	*P. vulgaris* seeds +gilbenclamide	Diabetic rats	No side effects	Potent hypoglycemic and antihyperglycemic activity
[99]	Bean flour crude	Diabetic rats	No side effects	No change in physiological parameters, in biochemical marker and organ weight
[100]	Aqueous extract	Healthy rats	No side effects	No significant weight loss, change in organ weight, food consumption and blood biochemical parameters.

Table 1.1 Main studies on animal models to test the efficacy of P. vulgaris extract

These results have laid the foundations for studying the role of beans extracts on human being with particular regard to the safety of the administered product.

1.3.2 Early studies on humans

The α-amylase inhibitor from *P. vulgaris* powder has also been tested on human models. Many of the early studies carried on in 70s and 80s on the bean extract inhibitor are discussed in different reviews [2,38,103].

In the first studies crude white bean-based extracts were used. The results were disappointing; no decrease in human starch digestion was observed because of insufficient inhibitor activity and instability [104–106].

A series of subsequent and more promising studies were published by a research group from the Mayo Clinic (Rochester, Minnesota, USA) in the 1980s using a partially purified white bean product [107,108].

In vitro it was found that the partially purified inhibitor inactivated intraduodenal, intraileal and salivary amylase without being affected by exposure to gastric juice [107]. These findings were also confirmed *in vivo*, by the reduction of amylase activity in the duodenal, jejunal and ileal intralumen after the administration of partially purified amylase inhibitor accompanied by a significant reduction of gastric inhibitory polypeptide (GIP) concentration [108].

These results were later confirmed by other authors that observed as the partially purified extract significantly reduced absorption of complex carbohydrates from the terminal ileum and decreased GIP concentration [109,110].

In a series of studies several doses of partially amylase inhibitor were administered both healthy and diabetic subjects. A reduction in starch digestion and in postprandial glucose, C-peptide, insulin and GIP level was recorded [111,112]. The only side effect was

represented by a temporary diarrhea and the authors suggested that impurities in the partially purified preparation may be responsible for this side effect.

Similar results were obtained by Layer et *al.*, testing 13 subjects (8 healthy volunteers and 5 subjects with type 2 diabetes). The effect of a partially purified bean-derived amylase inhibitor was recorded on the ingestion of 50 g of a test meal made of rice, cocoa and aspartame. Also in this case, a decrease in postprandial plasma glucose and insulin concentration in both healthy and diabetics subjects was observed [113].

In 2000, the first study was conducted to investigate the effectiveness of white bean extract in weight loss [114].

An inhibitor product (Suco-Bloc®) tablet (containing 200 mg of white kidney bean extract, 200 mg of inulin and 50 mg of *Garcinia cambogia* extract) was administered to 40 overweight healthy subjects (BMI between 27.5 and 39).

In this randomized, double-blind, placebo-controlled trial the subjects had to eat 2 inhibitor tablets after all 3 meals per day for 12 weeks. After 3 months a significative weight loss, a reduction of BMI and a decrease of body fat percentage in the tested group were recorded. No sides effects were recorded in the experiments.

These papers confirmed the preliminary results obtained in the animal models. Not only the inhibitory activity is unaffected by gastric juices, but it reduces the absorption of complex carbohydrates, lowers blood glucose concentration, C-peptide and GIP levels, with almost negligible undesirable effects.

Moreover, the extract effectiveness in weight loss laid the groundwork for the study of commercial products that could be used as dietary supplements in the fight against obesity and diabetes. Further experiments became, in fact, necessary to improve purifications techniques and to test the safety of the product.

1.3.3 PHASE2®: commercial products

Subsequent studies have focused on using the starch blocker IQP-PV-101, known commercially as PHASE2®, but previously named Phaseolamin 2250, because one gram of the product blocked 2,250 starch calories [115]. It is also marketed globally under the Starchlite® and PhaseLite® brands [116].

It is a dried aqueous extract from the common white bean *P. vulgaris* (Pharmachem Laboratories; Kearny, NJ, USA) produced from non-GMO whole kidney beans and certified as gluten free. It is used as a dietary supplement (odorless and tasteless) in various forms, including powders, tablets, and capsules.

Each lot of PHASE2® has at least 3000 α-amylase inhibiting units (AAIU) per g when tested at a pH 6.8 using potato starch as substrate and pancreatin as enzyme source [2].

As declared in the Evaluation of the Generally Recognized as Safe (GRAS) status of PHASE 2® white bean (*Phaseolus vulgaris*) extract, a typically recommended dose is 1-2 capsule containing each 500 mg of PHASE 2®, taken 3 times a day during daily meals, or 1500-1300 mg per day [117]. Heat treatments inactivates the hemagglutinating power and trypsin activity, thus ensuring the product safety [118].

1.3.4 Meta-analysis on clinical trials

Many clinical studies about PHASE 2® are collected in two meta-analyses. The most recent has been proposed by Udani and coworkers, investigating the effectiveness of PHASE 2® in reducing body weight and fat [12].

They only included in their analyses those studies that met the main requirements collectively termed PICOS (Population, Intervention, Comparison, Outcomes and Study)

criteria: Participants had to be obese or overweight; Intervention had to be done with PHASE 2®; the Control group had to be compared with the test group; Outcome measurements had to include measurements of body or fat mass; and Study design had to be based on double-blind, placebo-controlled parallel or crossover trial, randomized or open-label studies.

Based on these characteristics they selected 11 studies for meta-analysis for weight loss with a total of 573 subjects and 3 studies for meta-analysis for fat loss with a total of 110 subjects.

They found that the intake of PHASE 2® resulted in a weight loss difference of 1.08 kg and in a fat reduction of 3.26 kg.

These conclusions partially contradict what was previously stated by Onakpoya et *al.* in a previous meta-analysis [119]: in fact they did not find any significant difference in weight loss between treated and control group.

As suggested by Udani et *al.*, this difference may be due to the choice to include in the Onakpoya meta-analyses all studies on *P. vulgaris*, and not only on PHASE 2® [12]. These differences could have led to the inclusion of studies based on different partially purified preparations. However, also the meta-analysis from Onakpoya et *al.* showed a significant effect on fat loss, confirming a sharper effect on this body parameter.

1.3.5 Studies on weight loss in humans

Many authors focused their attention on testing the effectiveness of PHASE 2® in the loss of body weight and body composition (as summarized in Table 1.2).

In a series of papers, Udani and coworkers investigated the role of PHASE 2® in decreasing body weight, triglyceride level and reducing waist size.

They did not achieve a significant statistical difference between active and placebo group in weight loss, by lowering triglyceride level and reducing waist size, but they underlined the potential of PHASE 2® in the treatment of obesity and hypertriglyceridemia [120,121].

Different conclusions were reached by Celleno et *al.* [122].Their results showed that the extract of *P. vulgaris* significantly contributes to reduction of body weight, BMI, fat mass, adipose tissue thickness, and waist, hip and thigh circumferences in overweight treated subjects. At the same time the lean body was preserved.

A similar study was carried out in 2010 on overweight men and women [123] in which a statistically significant difference was recorded in reduction of body weight and waist circumference in an active group compared to a placebo group. On the other hand, no significant change in hip circumference was recorded.

More recently PHASE 2® was tested for its efficacy in weight loss and weight maintenance in subjects with BMI between 25 and 35 kg/m^2 [116].

This research is of particular importance for the sample size of the Weight Loss (WL) trial and for the duration of Weight Maintenance (WM) study (the largest sample, 123 subjects, and the longest single clinical trial, 24 weeks, respectively) conducted on *P. vulgaris* extract.

It proves the efficacy of the product in WL after 12 weeks (2.91 ± 2.63 kg vs. 0.92 ± 2.00 kg, $p< 0.001$) and in WM after 24 weeks (the mean weight of the subjects at week 24 was $99.34\pm2.96\%$ of baseline weight).

Ref.	Duration	Subjects	Dose of Phase 2®	Main results	Trial type
[120]	8 weeks	n=39	1500 mg twice a day	No significant difference in WL and triglyceride level	Randomized, double-blind, placebo-controlled
[121]	4 weeks	n=25	1000 mg twice a day	No significant difference between in WL and reducing waist size	Double-blind, placebo controlled
[122]	4 weeks	n=60	445 mg	Statistically significant difference in reducing BW, BMI, fat mass, adipose tissue thickness, and waist, hip, thigh circumferences. Lean body preserved.	Randomized, double-blinded, placebo-controlled
[123]	8 weeks	n=101	2000 mg	Statistically significant in reduction in BW and waist circumference. No significant change in hip circumference.	Randomized, double-blinded, placebo-controlled
[116]	12 weeks	n=123	1000 mg three times a day	Statistically significant difference in WL, waist circumference and BMI reduction.	Randomized, double-blinded, placebo-controlled
[116]	24 weeks	n=49	1000 mg three times a day	73.5 % of WM	Randomized, double-blinded, placebo-controlled
[124]	35 days	n=120	2400 mg	Statistically significant difference in BW, BMI, fat mass, adipose tissue thickness, and waist circumferences.	Randomized, double-blinded, placebo-controlled

Table 1.2 main clinical trials reported on humans for PHASE2® preparations. Abbreviations: BW, body weight; BMI, body mass index; WL, weight loss; WM, weight maintenance.

The growing interest in this research field is emphasized by a very recent paper that studies WL in obese subjects after the intake of 2,400 mg of *P. vulgaris* extract before each daily meal in a short period (35 days) [124]. The differences in WL between the active and placebo group were significant ($p < 0.1$; 2.24 Kg vs 0.29 Kg). BMI decreased by an average of 0.79 kg/m^2 and the body fat decreased by 1.53% on average compared with the baseline. Other recorded parameters were thickness of subcutaneous fat, waist and hip circumference that significantly decreased after the treatment.

These studies demonstrated the effectiveness of the commercial product PHASE 2® in the treatment of obesity. Weight loss, reduction in BMI and decrease in waist circumference are the main effects of taking this food supplement.

However other important issues have been investigated to evaluate the effect of *P. vulgaris* extract in reducing postprandial plasma glucose, hyperglycemia and insulin response.

1.3.6 Effect of α-AI1 on hyperglycemia and hyperinsulinemia in humans

One of the obesity-related diseases is diabetes mellitus, a metabolic disorder characterized by chronic hyperglycemia. Many therapies have been proposed by the scientific community to treat type 2 diabetes; the use of α-amylase inhibitor is one of the effective therapeutic strategies to lower postprandial blood glucose levels [125]. In fact, as summarized in Table 1.3, the use of α-amylase inhibitors to reduce hyperglycemia and hyperinsulinemia had already been studied since 1973 on rats, dogs, and healthy humans [126].

As mentioned above, both Boivin and Layer groups showed that a partially purified amylase inhibitor was able to decrease postprandial plasma glucose and insulin concentration in both healthy and diabetic subjects [111,113].

More recently, a new commercial supplement produced with the intent of preventing obesity and diabetes named "Super Bows Diet Type B" has been tested in Barrious Laboratories [127].

This granular supplement contains PHASE 2®, *Coleus forskohlii* extract and mushroom chitosan.

In a double-blind crossover test including 13 men and women with a fasting blood-glucose level above 126 mg/L, the active product or the placebo (in a single dose) were administered 5 minutes before eating 300 g of polished rice.

The analysis of blood glucose levels measured before and after eating (30-120 minutes later) showed that blood sugar levels and insulin levels were significantly lower in the active group as compared to the placebo group measured respectively 30 minutes and 30-60 after meal intake. Unfortunately, these results have not been published in a peer-reviewed journal.

On the other side, in 2009 three different independent papers focused on the same topic were published.

In the first study differences in Glycemic Index (GI) were investigated after the intake of white bread and butter with and without the addition of PHASE 2® [128]. The GI is a scale that represents the rise of glucose level after food intake and is defined as 'the incremental area under the blood glucose response curve of a 50 g carbohydrate portion of a test food expressed as a percent of the response to the same amount of carbohydrate from a standard food taken by the same subject'.

PHASE 2® was administered to 15 randomized volunteers with BMI between 18 and 25 (only 13 were considered in the final analysis) in a capsule or powder form at the dosage of 1500 mg, 2000 mg, and 3000 mg. The GI was calculated using standard capillary glucose measurements.

The capsules with 2000 mg and 3000 mg of PHASE 2® reduced GI significantly, while the powder formulation was effective only with the highest dose administration.

The second study included two different placebo-controlled, cross-over tests [129].

In the first trial 4 slices of white bread and 42 g of margarine with or without 1500 mg of PHASE 2® were administered to 11 normoglycemic subjects. With a clinical procedure, the blood was drawn before the meal and after every 15 minutes for 2 hours. A faster return to the plasma glucose base line (62 vs 80) was found in the active group compared to the placebo group and it was suggested that only 1/3 of the carbohydrates in the bread were absorbed with PHASE 2®.

In the second trial 7 subjects consumed a frozen dinner of country fried steak with gravy, mashed potatoes, green beans, and cherry-apple pie with or without a lower PHASE 2® dose (750 mg).

Blood was sampled every 10 minutes for 60 minutes then periodically until 2 hours.

Also in this case, the active group returned to the baseline faster than untreated subjects (58 vs 70 min) and only 2/3 of the carbohydrates was absorbed using PHASE 2®.

In the third study the hypoglycemic power of bean extract was also investigated in combination with acarbose on 12 healthy volunteers. The subjects were administered 1500 mg *P. vulgaris* extract and 50 mg acarbose, or acarbose 50 mg alone, before a carbohydrate meal. However, no significant differences were found between the two groups in reducing postprandial glucose level [130].

In a more recent trial the efficacy of *P. vulgaris* extract (Beanblock; Indena S.p.A.) was investigated both on glycometabolic and appetite control [131].

In this double-blind, randomized, cross-over study, to 20 healthy fasting volunteers were asked to eat a standardized meal consisting of a sandwich of white bread, ham, oil and tomato with 100 mg of active or placebo tablet. The blood was drawn at baseline and periodically for three hours after meal to record glucose, insulin, C-peptide and ghrelin concentration.

The intake of the bean extract reduced the glucose response to the meal, accompanied by a lower insulin level and C-peptide excursions. The sense of satiety was significantly reduced in the placebo group compared to the active one (third hour after meal) as a result of the suppression of ghrelin secretion inducing a lower desire to eat in the active group. Several studies have proved the efficacy of *P. vulgaris* extract in reducing postprandial blood glucose concentration and so it is suggested as a valuable tool to prevent the onset of diabetes [132].

PHASE 2® is effective in reducing blood glucose and insulin levels in both healthy and diabetic subjects confirming what has been observed in preliminary animal studies and early human trials. As noted above, the side effects of this supplement are mostly attributable to transient or even absent episodes of diarrhea.

However, more studies are necessary to explore this topic in more depth in order to resolve some discrepancies among the findings. This research field is in any case still open and the consumption of beans has even been proposed as a possible low-cost approach to reduce some ophthalmic disease such as cataract in patients with type 2 diabetes [133].

Ref.	Administration	Subjects	Side effects	Main results
[113]	Partially purified extract	Healthy and diabetics	Abdominal discomfort and diarrhea	Decrease in postprandial plasma glucose and insulin concentration in both subjects.
[111]	Partially purified extract	Healthy	Diarrhea	Reduction in starch digestion and postprandial glucose
[127]	PHASE 2®+*Coleus forskohlii* extract+mushroom chitosan.	Diabetic	No side effects	Reduction in blood sugar levels and insulin levels
[128]	PHASE 2®	Healthy	No side effects	Reduction of GI after the administration of 2 and 3 g of PHASE 2®
[129]	PHASE 2®	Healthy	No side effects	A faster return to the plasma glucose base line after a meal
[130]	PHASE 2®+ acarbose	Healthy	Not available	Reduction in postprandial glucose concentrations
[131]	Partially purified extract	Healthy	No side effects	Reduction in glucose, insulin and C-peptide levels. Reduce the desire to eat, prolonging the sense of satiety.

Table 1.3 Main clinical trials on humans for the treatment of hyperglycaemia and hyperinsulinemia with P. vulgaris extract

1.4 Safety

It should be noted that beans and their extracts also contain anti-nutritional compounds. Trypsin inhibitors, lectins such as phytohemagglutinin, polyphenols (condensed tannins and anthocyanins) and some oligosaccharides, in fact, counteract the beneficial effects deriving from their consumption [134]. The presence of these substances may interfere with digestibility and availability of nutrients [135]. Many studies have reported the occurrence of side effects and risk of adverse reactions after the ingestion of raw beans in humans and animals [136–138].

Particular attention was given to PHA that, if administrated at high concentrations, causes hyperplastic growth of the small intestine, lower fractional rate of protein synthesis in skeletal muscle and overgrowth of *Escherichia coli* in the lumen in dose-dependent manner [139,140].

With the aim of removing the activity of antinutritional compounds, different techniques have been reported and summarized by Kumar and coworkers in a recent review [141]. For example it has been observed that PHA is inactivated by cooking [142–144]. Unfortunately, this treatment also inactivates α-AIs.

Furthermore, since hemagglutination activity greatly differs among all cultivars [145,146] and the α-amylase inhibitor is more active on some than others [32,81,147,148], it is crucial to choose a cultivar with less PHA concentration and more α-amylase activity as a raw material to produce bean extract.

However, the role of PHA in body weight loss is still debated. In fact, the intake of raw bean extract mixed in the diet with PHA lower than 0.2 g/kg leads to the reduction in body fat and body weight in obese Zucker rats [149]. This group lost more fat compared with the control group without loss of skeletal muscle and body proteins.

A higher concentration (0.4 g/kg) of PHA was tolerated if accompanied by a higher lipid dose in the diet.

Based on this evidence bean lectin has been suggested as a dietary adjunct to tackle obesity in a safe and effective dose-range. However, when rats were fed with a high concentration of PHA and low in α-AIs, there was no hypoglycemic effect [150].

Therefore, the attention has turned to the preparation of *P. vulgaris* extract with a dual action: i) inhibition of α-amylase and ii) anorexigenic effect caused by PHA [93].

PHASE 2® is instead prepared using a special process to inactivate hemagglutination power and trypsin inhibiting activity and many studies tested its safety. The finished product contains less than 700 hemagglutinating activity units (HA) per g.

Acute and subacute toxicity on rats was investigated by several studies [118,151,152]. No significant side effects or mortality was recorded in these trials. The toxicity level was considered acute when it exceeded the highest dose tested in acute administration corresponding to 5 g PHASE 2®/kg BW [38].

Moreover, the absence of side effects related to the presence of PHA confirmed the product safety.

The effects of subchronic (30 days treatment) and chronic (24 weeks) of PHASE 2® administration were studied on humans too, and no side effects were recorded [116,122]. Despite most studies are focused on PHASE 2®, it is not the only α-amylase inhibitor commercially available.

In 2020 another carbo-blocker extracted from *P. vulgaris* called Max Bloc® was tested [153]. Compared with three other commercial carbo-blockers from China and the United States it has a α-amylase inhibitory activity 10-16 times higher and no hemagglutinin toxicity (0 HAU/g vs 256,000-640,000 HAU/g of the others).

Moreover *in vitro* studies have shown that the ingestion of Max Bloc® reduces starchy food digestion by 69.2% to 93.3%.

Based on this evidence this supplement has been proposed as a valid candidate in weight management and glucose control.

On the whole, α-amylase inhibitor commercial products showed different characteristics probably linked due to the different extraction procedures. These could also affect the real dose administered and the resulting effectiveness on the patient. The joint action of α-amylase inhibitor and PHA may improve the effectiveness of the product in a dose-dependent manner. Nevertheless, many of these studies look preliminary, thus more experiments should improve the safety and efficacy of these commercial products.

1.5 The modulation of gut microbiota

The increased awareness of the role of gut microbiota in human and animal health is leading to more investments and research on this topic [154]. It has been demonstrated that the diet plays a preponderant role in the modulation of microbiota, even more important than genetic factors [155].

It has been proved that the gut microbiota composition changes in lean and obese individuals thus revealing that obesity is associated with the reduction in the abundance of *Bacteroidetes* and with the increase in the abundance of *Firmicutes* [156].

Based on this evidence some recent studies have focused their attention on the effects that *P. vulgaris* extracts may have on microbiota composition in mice [95,157].

Shi et *al.* investigated the role of α-amylase extract in body weight, serum lipid levels, and gut microbiota composition in obese rats. During the experiment three different doses of extract (0.5%, 1%, and 1.5%) were added to the diet and administered to induced-obese

rats for 10 weeks. At the end of experiments rats fed with the two highest doses of extract showed a significantly lower BW. Under these conditions, abdominal fat accumulation, serum total cholesterol and serum LDL levels were significantly reduced.

Interesting considerations have been made about the relationship among colonic content, pH, and the concentration of Shorty Chain Fat Acid (SCFA). The administration of medium and high dose of bean extract significantly increased the colonic content compared to obese and non-obese rats and led to a decrease in pH values of the colonic content [95].

This pH reduction is closely related to the increase of SCFA in intestinal content arising from the fermentation of carbohydrate by gut microbiota [158,159]. The authors underlined the importance of SCFA in metabolic health, recalling that propionic acid (increased by medium and high doses) has an important role in the reduction of lipids level [160] thus suggesting that the lipid-lowering effects resulting from the administration of *P. vulgaris* extract may be related to this increase in propionic acid.

Important changes were also recorded in gut microbiota after the administration of different diets. The high fat diet of the mice involved a reduction of diversity and richness in gut microbiota composition. However, the administration of *P. vulgaris* extract involved a reduction of the relative abundance of *Firmicutes* and *Proteobacteria*, and an increase of the relative abundance of *Bacteroidetes*, *Butyricoccus*, *Blautia* and *Eubacterium* (phylum level). The latter groups of bacteria are SCFA-producing, protecting the intestinal gut from damage preventing the onset of obesity and related diseases.

These conclusions are in agreement with what found previously by Song et *al*. in the first study on the change in gut microbiota in diet-induced obese mice by administration of

kidney beans extract [157]. Similarly, rats have been divided into three groups (fed with low fat diet, high fat diet and high fat diet added with *P. vulgaris* extract) to monitor the body weight, serum lipid level, hepatic steatosis, and change in gut microbiota composition for 14 weeks.

Even in this case, the *P. vulgaris* extract treatment reduced body weight and food intake compared to animals fed with only high fat diet. The high fat diet involved an increase in total cholesterol (LDL, HDL), and serum triacylglycerols, while the administration of bean extract led to a reduction TG, TC, LDL but not HDL. The bean extract turned out to have an anti-hepatosteatosis effect and to cause a decrease in glucose blood levels in rats fed with a high fat diet.

In accordance with the other study, the high-fat diet induced a significance increase of *Firmicutes* and decrease in *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia*, while bean extract involved a decrease in the relative abundance of *Firmicutes*, but a significant increase in *Verrucomicrobia* and *Actinobacteria*. The increase of the relative abundance of *Bifidobacterium*, *Lactobacillus* and *Akkermansia* was also recorded at the phylogenetic genus level.

So, it is evident that the subjects receiving the extract benefit both in terms of weight body loss, reduced blood glucose level and intestinal health.

In addition, Shenil Wang et al. investigated the modulation of gut microbiota in high-fat fed C57BL/6J mice after 8 weeks of administration of *P .vulgaris* extract, Yogurt and a mixture of *P. vulgaris* extract and yogurt (YPVE) as additive feeding [161].

At phylum level the *Bacteroidetes* and *Proteobacteria* in YPVE group was declined comparing to the other groups while the abundance of *Firmicutes* in YPVE group remained higher than high-fat fed mice. The YPVE mice microbiota was characterized

by richness and abundance of microbial species suggesting that the YPVE led to a healthy direction on the regulation of intestinal flora.

In summary, the intake of *P. vulgaris* extract in mice has the potential to modulate gut microbiota. The microbiota of treated animals was characterized by a greater richness in species. It was recorded an increase of the relative abundances of *Bacteroidetes* and *Akkermansia* and a reduction of *Firmicutes* and *Proteobacteria,* with the proliferation of SCFA-producing bacteria which reflects positively on the well-being of the host. These findings are in fact giving further impetus to research in this field. Many recent works are focused on the role of *P. vulgaris* intake in microbiota modulation in animal models [162–164].

The role of *P. vulgaris* extract in the modulation of gut microbiota has been tested also on human being recently. Type 2 diabetes patients were treated with white common bean extract and monitored for 4 months. It was found that the abundances of *Bifidobacterium*, *Faecalibacterium* and Anaerostipes were higher in the treated group, and the abundances of *Weissella*, *Klebsiella*, Cronobacter and *Enterobacteriaceae*_unclassified were lower than those in the control group after *2* months. The *Bifidobacterium* remained more abundant in the treated group also after 4 months [165]. The modulation of microbiota with the increase of SCFA-producing bacteria, would explain why in treated patients it was observed a decrease of diabetic complications.

Other experiments are needed to confirm these data and standardize the treatment plan.

1.6 Taxonomy and phylogenetic classification of *Phaseolus vulgaris*

The choice to use the common bean seeds as the starting material for this study stems from the fact that this is one of the most widely consumed leguminous plants worldwide.

It is good source of protein fiber and bioactive compounds, including α-AI and α-glucosidase inhibitor [166].

The high content in protein, vitamins, certain minerals (Ca, Fe, Cu, Zn, P, K, and Mg) and carbohydrates makes it a fundamental element in the diet of millions of people above all in underdeveloped and developing countries [167].

It is believed that the genus *Phaseolus* originated 4 to 6 million years ago in MesoAmerica [168].

After the discovery of America, the bean spread all over the world: Europe, Mediterranean region, (mostly eastern) Africa and parts of Asia [169].

This spread has led to the coining of different names in different parts of the world such as, only in English, Common bean, bean, French bean, kidney bean, runner bean, snap bean, string bean, garden bean, green bean, haricot bean, bush bean, navy bean, pole bean, flageolet bean.

P. vulgaris is a member of *Fabaceae* family (Table 1.4), which comprises species displaying a wide variety of forms. *P. vulgaris* shares many of the features characterising the family, but two ones are unique of the genus: the keel of the flower terminates in a coil, having from one to two turns and uncinate hairs are present on both vegetative and reproductive structures of the plant [170].

It is a highly polymorphic warm-season, herbaceous annual. The leaves, borne on long green petioles, are green or purple in colour and trifoliate.

The inflorescences are axillary or terminal, 15-35 cm long racemes. The flowers are arranged in pairs or solitary along the rachis, white to purple and typically papillonaceous. After the pollination from the flower come one pod. Pods could be slender, green, yellow, black or purple in colour, sometimes striped and contain 4 to 12 seeds. The seeds length

ranges from 0.5 to 2 cm long; different varieties have highly variability in colour: white, red, green, tan, purple, grey or black.

Kingdom	Plantae
Subkingdom	Viridiplantae-green plant
Infrakingdom	Streptophyta- land plants
Superdivision	Embryophyta
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Rosanae
Order	Fabales
Family	Fabaceae-peas,legumes
Genus	Phaseolus L.-bean
Species	*Phaseolus vulgaris*

Table 1.4 Taxonomic classification of P. vulgaris

Phaseolus vulgaris cultivation is also widespread throughout Italy and Sardinia.

To valorize our biodiversity, it has been selected nine uncommon Sardinian cultivars and one Italian cultivar as reported in Table 1.5.

Faita Sorgonese, Granino, Fasolu pintau, Fasolu, Fazadu Nieddu were collected by the Interdepartmental Centre for the Conservation and Enhancement of Plant Biodiversity (CBV) of the Sassari University, as part of a project that involved researching in numerous centers in Sardinia the bean varieties still cultivated in family vegetable gardens by elderly farmers.

Common name	Place	Source	Accession number
Fasolu pintau	Sardinia, Sadali	CBV	ACC. 113
Fasolu	Sardinia, Belvì	CBV	ACC. 20
Pisu Grogo	Sardinia, Austis	LAORE	ACC. 152
Faitta a Cavanedda	Sardinia, Tiana	LAORE	ACC. 147
Faitta Sorgonese	Sardinia, Tiana	CBV	ACC. 1
Granino	Sardinia, Tempio	CBV	ACC. 28
Fazadu Nieddu	Sardinia, Pattada	CBV	ACC. 121
Bianco	Sardinia, Fluminimaggiore	AGRIS	ACC. 124
Nieddone	Sardinia, Ploaghe	AGRIS	ACC. 177
Lamon	Veneto, Belluno	Commercial	BBG-PV.1

Table 1.5 Specificities of P. vulgaris cultivars included in the study

Pisu grogu and Faita Cavanedda were instead collected by the Regional Agency for the implementation of agricultural and rural development programs (Laore).

The two cultivars Bianco di Fluminimaggiore and Nieddone were collected by the Regional Agency for Agricolture (Agris) within the project RISGENSAR.

All these cultivars came from small plots of land, they are rare and not yet characterized for their alpha amylase inhibitory activity.

Meanwhile, Lamon cultivar comes from Belluno province, and it is marketed as Lamon bean cultivar from the Belluno Valley. It is recorded with the mark Protected Geographical Indication (IGP) and represents the Italian commercial standard (http://www.fagiolodilamon.it/en/).

Figure 1.6 Samples of P. vulgaris cultivars

Maintaining these rare varieties and their degree of purity is possible thanks to the bean's autogamy [171], but the role of organizations that safeguard biodiversity is essential.

1.7 Aim of the work

Based on this evidence and on the data reported in literature the study is divided into two chapters.

The aim of Chapter 2 deals with the comparison among 10 *P. vulgaris* cultivars.

Firstly, an assay to quantify the activity of α-amylase and α-glucosidase enzyme has been developed. This method is used to make a comparison of the inhibitory power of the raw extract of the examined cultivar against α-amylase and α-glucosidase enzymes. Thus, the content in α-amylase and α-glucosidase inhibitors have been quantified and compared.

Then the hemagglutination power has been investigated to identify the most promising cultivars cultivars with lower antinutritional factors content.

The gene encoding for the α-amylase inhibitor was also and sequenced in all cultivars examined. The following subjects will be boarded in this section:

- identify phylogenetic relationship among cultivars,

- check whether mutations are present, how they affect inhibitory activity,

- improve the biodiversity knowledge about these poorly studied common bean cultivars.

A recently developed complexity-reduction quantum mechanical (QM-CR) approach, based on complexity reduction of density functional theory calculations, has been also employed to characterize biologically relevant intermolecular interactions and in particular between the α-amylase enzyme and α-AI.

In Chapter 3 the extraction and the purification of α-amylase inhibitor will be performed in the most promising cultivar(s).

Several extraction techniques are going to be evaluated and a purification protocol is going to be developed. The inhibitory activity of purified will tested on different amylase

enzymes and α-glucosidase. After these steps the purified inhibitor is going to be characterized from a biochemical point of view. It is going to be evaluated its stability to different pH and temperature conditions and its kinetics.

All these experiments aim to identify the cultivar most promising to produce nutraceutical supplements. The commercial value of these cultivar could be increased thanks to these discoveries.

2. Biochemical and phylogenetic analysis of Italian *Phaseolus vulgaris* cultivars as sources of α - amylase and α-glucosidase inhibitors

The increasing incidence of obesity and diabetes is defined as an epidemy by the World Health Organization [172]. The obesogenic environment of the modern society led to the spread of these health conditions [173]. Many therapeutic approaches have been proposed to tackle the spread of these diseases such as the improvement of physical exercise, the reduction of the carbohydrates in the diet, and the intake of food supplements and in particular of phytochemicals that could reduce the carbohydrates absorption [174]. Among the food supplements, some based on proteinaceous α-amylase inhibitors from common bean *P. vulgaris* are already present in commerce [166].

In this respect, 9 poorly studied common bean cultivars from Sardinia (Italy) are investigated for their inhibitory power against digestive enzymes, and their biological activity has been compared to a well-known commercial bean variety from Veneto (Italy): Lamon.

Moreover, to better understand the differences in the inhibitory activity, phylogenetic studies have been carried out on the inhibitor gene to reconstruct the aminoacidic sequence of the protein inhibitor from all cultivars.

2.1 Materials and methods

2.1.1 Chemicals

2-Chloro-4-nitrophenyl-α-D-maltotrioside (93834-100MG), *p*-nitrophenyl-α-D-glucopyranoside (487506-1GM), α-amylase from porcine pancreas (Type VI-B A3176-2.5MU), α-Glucosidase from *Saccharomyces cerevisiae* (Type I G5003-1KU) and α-amylase from *Bacillus licheniformis* (A4551-100MG) were purchased from Sigma–

Aldrich (Milan, Italy). All other reagents used were of the highest grade available, purchased from Sigma–Aldrich (Milan, Italy) and used without further purification.

2.1.2 Plant materials

The cultivars included in this study are part of a wider germplasm collection by the Sardinian Agricultural Agency (AGRIS, Cagliari, Italy), by the Regional Agency for the implementation of agricultural and rural development programs (LAORE) and in the Centre for the Conservation and Enhancement of Plant Biodiversity (CBV; Sassari; Italy). 9 common beans (*Phaseolus vulgaris* L) were included collected in all the region of Sardinia, mainly deriving from local small farms and differentiated by morphologic features (different shape, dimension and color), as shown in Table 1.5 and Figure 1.6. One Venetian commercial cultivar (Lamon) was also included in the work as a comparison model. Lamon was purchased from the local Consortium for the protection of the Protected Geographical Indication (http://www.fagiolodilamon.it/en/).

As an experimental positive control, a commercial food supplement was purchased from a local market. The supplement was based on a purified common bean extract. Each tablet contained 500 mg of extract and was processed using a similar protocol of *P. vulgaris* seeds.

2.1.3 Protein extraction

Legume seeds of the different cultivars were homogenized to a fine powdery flour in a blender. The flours were then immediately extracted and used for the extraction. Briefly, aliquots of 2 grams of flour were suspended in 10 mL Bis-Tris buffer pH 6.5 mM, NaCl 0.1 M; then stirred for 1 hour and centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was used to record biological activities.

Total proteins were measured by the method of Bradford, using bovine serum albumin as standard [175].

2.1.4 α−Amylase inhibition assays

Two α-amylase inhibitory activity assays were performed.

The first one was based on the determination of reducing sugars produced by α-amylase action using 3,5-dinitrosalicylic acid (DNS) [29]. A typical amylase assay mixture consists of 5 EU amylase enzyme and an adequate amount of sample in phosphate buffer (pH 6.9) in a final volume of 375 μL. After 10' at 37°C, the reaction was initiated by the addition of 125 μL of 1% soluble starch at 37°C. The reaction was stopped after 10 min by the addition of 250 μL 3,5-dinitrosalisylic acid (DNS) reagent [176]. After incubation in boiling water bath for 5 min, the contents of the test tubes were cooled and diluted 1:10 with distilled water. Absorbance of the mixture was measured at 540 nm using UV-vis spectrophotometer (UltroSpec 2100 pro, Amersham Bioscience, Milan, Italy), using proper controls in the absence of enzyme, sample, or substrate. The amylase inhibitory unit (IAU) was defined as the number of amylase units inhibited under the assay conditions.

The inhibition of α-amylase activity was also determined using 2-chloro-*p*-nitrophenyl-α-D-maltotrioside (CNP-G3), as previously described with minor modifications [177]. A reaction mix containing 250 mM sodium phosphate buffer at pH 6.5, NaCl 60 mM, $CaCl_2$ 5 mM, KSCN 500 mM, and 5 EU α-amylase from porcine pancreas (unless otherwise stated) was used. The solution was mixed and incubated in absence or presence of samples at 37°C for 10 min, in a final volume of 150 μL. After incubation, 50 uL of a 9 mM CNP-G3 solution was added and the amount of 2-chloro-*p*-nitrophenol released by the enzymatic hydrolysis was monitored at 405 nm in microplate spectrophotometer UV/VIS

MultiskanGo (Thermo Fisher Scientific, Monza, Italy). Proper negative controls were performed in the absence of enzyme, sample, or substrate. As a positive control, a commercial food supplement extract (based on *P. vulgaris*) was added in each analysis.

One amylase E.U. was defined as the amount of enzyme capable of hydrolyze 1 μmol of CNP-G3 per minute at pH 6.5 and 37°C (monitoring CNP formation, $\varepsilon_{405} = 14{,}580$ M^{-1} cm^{-1}).

The amylase inhibitory unit (IAU) was defined as the number of amylase units inhibited under the assay conditions.

2.1.5 α−Glucosidase inhibition assays

α-Glucosidase inhibition assay used the same experimental conditions of CNP-G3 protocol. The substrate used was 2.25 mM *p*-nitrophenyl-α-D-glucopyranoside (pNPG).

One glucosidase E.U. was defined as the amount of enzyme capable of hydrolyze 1 μmol of pNPG per minute at pH 6.5 and 37°C (monitoring CNP formation, $\varepsilon_{405} = 14{,}580$ M^{-1} cm^{-1}).

The glucosidase inhibitory unit (IGU) was defined as the number of glucosidase units inhibited under the assay conditions. Proper negative controls were performed in the absence of enzyme, sample, or substrate. As a positive control, a commercial food supplement extract (based on *P. vulgaris*) was added in each analysis.

2.1.6 Hemagglutination assay

Human blood 2 mL was centrifuged at 2000 g for 3.5 minutes, supernatant was discarded, and the cell pellet was washed three times with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Red blood cells (RBC) pellet was suspended

to 50% (*v/v*) with PBS buffer, stabilized with 0.01% sodium azide and stored at 4°C. The life span of RBC was up to three weeks.

One aliquot of RBC 10% was prepared, and each raw extract was used at different serial dilutions (pure, ½, ¼, $^1/_8$, $^1/_{16}$….as far as $^1/_{512}$.)

For each combination of RBC 10% and protein concentration, the RBC sample was pipetted onto a glass microscopy slide and mixed in a 10 µL:10 µL ratio with the protein solution. The mixture was incubated for 5 minutes and observed by light microscopy (Digital Microscope Olympus BX81 (Olympus, Segrate, Italy). Images were captured with a charge-coupled device camera (Cohu, San Diego, CA, USA). Control samples were used by mixing 10 µLof the erythrocyte suspension with 10 µL of the PBS buffer was incubated with RBC. The degree of the agglutination was recorded in terms: no agglutination, negative reactions appeared as unchanged cloudy suspension; several large aggregates, visible aggregation of particles was seen in case of positive reaction.

In case of negative agglutination after 5 minutes, the incubation was extended up to one hour.

Results are expressed as the Minimum protein Concentration able to Agglutinate sample (MAC, mg/mL).

2.1.7 Statistical analysis

GraFit 7 (Erithacus Software, London, United Kingdom), R 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad INSTAT (GraphPad Software, San Diego, CA, USA) were used for data analysis. One-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test were used to assess the statistical significance of the differences. All the analyses were performed at least in triplicate

(unless otherwise stated), and the data are reported as mean ± standard error of the mean (SEM).

2.1.8 Biomolecular Assay: plant materials

Phaseolus vulgaris leaves obtained from the seeds described above were the starting material for biomolecular experiments on RNA. The tissue was treated with liquid nitrogen to obtain a thin powder and subsequently frozen and stored at –80 °C until use.

2.1.9 RNA extraction and reverse transcription

All the chemicals used in this study were purchased from Sigma-Aldrich (Merck Group, Milan, Italy) and were used without further purification. For the purification of total RNAs and PCR products, specific commercial kits were used as described below.

P. vulgaris leaves were the starting material for biomolecular experiments on RNA. The tissue was treated with liquid nitrogen to obtain a thin powder and subsequently frozen and stored at –80 °C until use.

As previously described [178] isolation of RNA from *P. vulgaris* and RT-PCR Total RNA was extracted from the leaves previously treated with liquid nitrogen using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's suggested protocol. The quality of purified RNA was verified by gel electrophoresis using a 1% denaturing agarose gel stained with SYBR Green II (Sigma-Aldrich), and the concentrations were measured using a NanoDrop 2000c UV-VIS Spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 260 nm. To obtain cDNAs, RNAs were reverse transcribed with

an oligo dT primer using an enhanced avian myeloblastosis virus reverse transcriptase enzyme (Sigma-Aldrich), following the manufacturer's recommendations.

Genomic DNA was isolated from freeze-dried plant samples using the Plant & Fungi DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol. The quality and quantity of the DNA were determined spectrophotometrically using a NanoDrop instrument.

2.1.10 PCR-CODEHOP Strategy

The CODEHOP (Consensus Degenerate Hybrid Oligonucleotide Primer) strategy consists of a method aimed at designing primers for PCR amplification of unknown gene sequences coding for proteins which have conserved important functional domains during evolution [179]. This is a strategy which, despite being developed for molecular phylogeny studies, is successfully used for the identification of unknown genes, coding for proteins with a sufficiently conserved structure. In accordance with the meaning of the acronym CODEHOP, the primers used in this methodology partially hybrids consisting of a non-degenerate 5' portion called clamp of about 18-25 nucleotides, and a 3'-OH degenerate portion called core of about 10-12 nucleotides. The primers are designed starting from the alignment of proteins of interest, expressed in different organisms, even if not phylogenetically related. The amino acid sequences are aligned with the Clustal Omega program (http://www.ebi.ac.uk/clustalo) which shows the degree of homology existing among the species considered.

```
CAD28835.1      MASSKLLSLALFLALLSHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSYNSYDS 60
CAH60260.1      MASSKLLSLALFLVLLTLANSATETSFNIDGFNKTNLILQGDAIVSSNGNLQLSYNSYDS 60
CAH60259.1      MASSKLLSLALFLVLLTLANSATETSFNIDGFNKTNLILQGDAIVSSNGNLQLSYNSYDS 60
                ************.**:.********* **.************* ****************
                                     F1 →                                    F2 →
CAD28835.1      MSRAFYSAPIRIRDST TGNVASFDTNFTM NIRTHRQANSAVGLDFVLVPVQPES KGDTVT 120
CAH60260.1      MSRAFYSAPIQIRDST TGNVASFDTNFTM NIRTHRQANSAVGLDFVLVPVQPKS KGDTVT 120
CAH60259.1      MSRAFYSAPIQIRDST TGNVATFDTNFTM NIRTHRQANSAVGLDFVLVPVQPKF KGDTVT 120
                **********:**********:*********************************: ******
                         →
CAD28835.1      VEFDT FLSRISIDVNNNDIESVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN 180
CAH60260.1      VEFDT FLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLSNPSTGKSN 180
CAH60259.1      VEFDT FLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVLAVSLLNPSTGKSN 180
                *****************:*************************::*** *******
                              ← R1                              ← R2
CAD28835.1      NVSTTVELEKE VYDWVSVGFSATS GAYQWSYETHDVLSWSFSSKFINL KDQKSERSNIVL 240
CAH60260.1      NVSTTVELEKE VYDWVSVGFSATS GAYQWSYETHDVLSWSFSSKFINL KDQKSERSNIVL 240
CAH60259.1      NVSTTVELEKE VYDWVSVGFSATS GAYQWSYETHDVLSWSFSSKFINL KDQKSERSNIVL 240
                *****************************************************************

CAD28835.1      NKIL    244
CAH60260.1      NKIL    244
CAH60259.1      NKIL    244
                ****
```

Figure 2.1. Multiple alignment of α-AI amino acids sequences from three different Phaseolus sources. The sequences were chosen from the GenBank SwissProt database and aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The figure shows the alignments on which the CODEHOP primers have been designed. An asterisk (*) denotes identical residues; double dots (:) represent a conserved residue substitution; a single dot (.) shows partial conservation of the residue. The arrow above the amino acid sequences indicates the position of the sense (F →) and antisense (← R) primers chosen from a group of candidate primers obtained from the CODEHOP program. The amino acid sequences chosen by the CODEHOP program for the design of the primers are in bold inside the boxes.

alignment (Figure 2.1), the sequences were pasted into a block of the motif finders using the EMBL-EBI search and sequence analysis tools [180]. The primers were designed using the default parameters of the j-CODEHOP server (https://4virology.net/virology-ca-tools/j-codehop/). The primers for the amplification of *P. vulgaris* cultivars α-AI cDNAs were chosen from a group of primer candidates provided by the j-CODEHOP programme (Table 2.1). Briefly, the first fragment of partial α-AI cDNA was obtained using the sense primer F2 (5'-GCTGGTGCCCGTGCAGCCCAAGTCCaarggngayac-3') in association with the antisense primer R2 (5'-TCAGCACGATGTTGGACCGCTCGGAyttytgrtcytt-3') (Figure 2.1).

CODEHOP Sequences	Comments

5'-CGGCAACGTGGCCACCTTCGACACCaayttyacnat-3'	F1 sense primer designed on the conserved peptide TGNVASFDTNFTM
5'-GCTGGTGCCCGTGCAGCCCAAGTCCaarggngayac-3'	F2 sense primer designed on the conserved peptide KGDTVTVEFDT
5'-AGGTGGCGGAGAAGCCCACGGACACccartcrtanac-3'	R1 antisense primer designed on the conserved peptide VYDWVSVGFSATS
5'-TCAGCACGATGTTGGACCGCTCGGAyttytgrtcytt -3'	R2 antisense primer designed on the conserved peptide KDQKSERSNIVL

Table 2.1 CODEHOP oligonucleotides used in PCR experiments and peptides chosen by the CODEHOP programme for the design of the primers

Each primer presents the consensus clamp given in upper case whereas the degenerate core is in lower case: y = [C,T]; r = [A,G], and n = [A,G,C,T]. PCR was performed in a solution containing 1.5 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3, 50 mM KCl, 200mM dNTP mix, 1 mM sense primer, 1mM antisense primer, 1μg of cDNA, and 1-3 units of Jump Start AccuTaq LA DNA polymerase mix (Sigma-Aldrich). Thermal cycles of amplification were carried out in a Personal Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using slightly different programs. The CODEHOP cDNA PCR products with the expected sizes were purified with a Charge Switch PCR Clean-Up kit (Invitrogen, Carlsbad, CA) and then sequenced.

2.1.11 cDNA Sequencing and Analysis.

cDNA sequencing was performed by Bio-Fab Research (Rome, Italy). Nucleotide and deduced amino acid sequence analyses were performed by means of freeware programs. Translation of nucleotide sequences was performed using the ExPASy translate routine software (http://ca.expasy.org/). Similarities were analysed with the advanced BLAST algorithm, available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), and with the FASTA algorithm version 3.0 from the

European Bioinformatics Institute website (http://www.ebi.ac.uk/fasta33/index).
Sequences were aligned with Clustal Omega.

2.1.12 Amino Acid Pattern and Profile Search

Potential N-glycosylation sites
For the detection of potential N-glycosylation sites, we used the tool *NetNGlyc* (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0). The tool considers that *N*-glycosylation is known to occur on asparagines which are localized in the Asn-Xaa-Ser/Thr stretch (where Xaa is any amino acid except proline). While this consensus tripeptide (also called *N*-glycosylation sequon) may be a requirement, it is not always sufficient for the asparagine to be glycosylated [181]. *NetNGlyc* attempts to distinguish glycosylated sequons from *N*- non-glycosylated ones. By default, predictions are only shown on Asn-Xaa-Ser/Thr sequons. The tool gives threshold and confidence values important for interpreting the results. Any potential crossing the default threshold of 0.5, represents a predicted glycosylated site. The potential score obtained is the averaged output of nine neural networks.

Potential Protein Kinase Phosphorylation Sites
For the detection of potential kinase phosphorylation sites, we used the tool *NetPhas-3.1* (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1). The tool predicts serine, threonine or tyrosine phosphorylation sites in eukaryotic proteins using ensembles of neural networks by default the server predicts phosphorylation sites for all serine, threonine, and tyrosine residues in the input sequences, displays all the predictions made for each such residue and generates graphical output illustrating the results [182].

2.1.13 GenBank Accession Numbers

The *P. vulgaris* cultivars α-AI gene sequences obtained in this study have been deposited in NCBI GenBank. as shown in Table 2.2.

2.1.14 Phylogenetic tree construction

The α-AIs partial cDNA sequences were submitted to phylogenetic analysis. The phylogenetic tree was constructed using the Mega 11 tool [183]. The evolutionary history was inferred using the Neighbor-Joining method [184]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed [185]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The consistency of the inferred phylogenetic tree was evaluated with bootstrap analysis of 500 replications.

Cultivar name and accession code	GenBank accession number	Gene name	Protein name
Faitta Sorgonese (ACC1)	OP779318	*pv1-α-AI*	PV1-α-AI
Fasolu (ACC20)	OP441067	*pv20-α-AI*	PV20-α-AI
Granino (ACC28)	OP329712	*pv28-α-AI*	PV28-α-AI
Fasolu Pintau (ACC113)	OP441066	*pv113-α-AI*	PV113-α-AI
Fazzadu Nieddu (ACC121)	OP441070	*pv121-α-AI*	PV121-α-AI
Bianco di Flumini (ACC124)	OP441071	*pv124-α-AI*	PV124-α-AI
Faitta a Cavanedda (ACC147)	OP441069	*pv147-α-AI*	PV147-α-AI

Pisu Grogu (ACC152)	OP441068	*pv152-α-AI*	PV152-α-AI
Nieddone (ACC177)	OP530245	*pv177-α-AI*	PV177-α-AI
Lamon	OP441072	*Pv-lam-α-AI*	PV-LAM-α-AI

Table 2.2: Cultivar names (with accession code), Genebank accession numbers, genes, and protein names.

2.1.15 Secondary structures prediction

The partial amino acid sequences coding for α-AI of each cultivar was subjected to secondary protein structure prediction by using Chou-Fasman method [186]. The tool used was available at http://cib.cf.ocha.ac.jp/bitool/MIX/.

2.1.16 Complexity-reduction quantum mechanical calculation

It was applied a recently developed approach for large-scale electronic structure calculations: complexity reduction in density functional theory (DFT) calculations [187,188], hereafter called complexity-reduction quantum mechanical QM-CR. This approach could allow to study and define protein/protein interactions, thus highlighting α-AI hotspots necessary for its inhibitory activity.

QM-CR differs from previous approaches in requiring no targeted parameterization or prior knowledge about the nature or sites of interactions, and it is based on full QM calculations on the entire system.

QM-CR leverages recent progress in computational chemistry [189] to handle tens of thousands of atoms in a single simulation. This enables us to capture and investigate biological processes involving several hundreds of amino acids.

Importantly, QM-CR can reveal the mechanisms behind intermolecular binding by decomposing interactions into chemical/short-ranged (which imply a shared electron) versus electrostatic/long-ranged (which do not involve shared electrons). We define as "hotspots" amino acids with a significant chemical contribution to the intermolecular interactions.

It was employed the BigDFT computer program [190] based on an ab initio DFT approach on a set of fully atomistic 3D structural models, to simulate intermolecular interactions of interest with a computational cost manageable on modern supercomputers. The approach employs the formalism of Daubechies wavelets to express the electronic structure of the assemblies in the framework of the Kohn–Sham (KS) formalism of DFT [190]. The electronic structure is expressed, by both the density matrix and the Hamiltonian operator, in an underlying basis set of support functions—a set of localized functions adapted to the chemical environment of the system.

The code provides efficient and accurate QM results for full systems of large sizes, delivering excellent performance on massively parallel supercomputers.

In the present study, it was employed the PBE approximation corrected by dispersion D3 correction terms [191] and Hartwigsen-Goedecker-Hutter (HGH) pseudopotentials [192]. Crystallographic structures are obtained from the RCSB database (RCSB PDB. [accessed 2021 Oct 23]. https://www.rcsb.org/) using PDB entries 1bvn, 1dhk and 1b2y.

The CheSS library [193] has been employed to calculate the system's density matrix. The calculations were performed at the gas phase showing the interaction energies at interface residues.

Starting from a representative 3D model of the molecules as our input, we calculate the system's electronic structure, from which we extract various quantities. We draw a contact

network to identify relevant chemical interactions among the spike RBD and the various interactors considered in this study. The strength of the inter-residue interaction is quantified by the "Fragment Bond Order" (FBO), a quasi-observable computed directly from the density matrix. More detailed description of this quantity can be found in literature. [194] The interaction is calculated using the electronic structure of the system in proximity of a given residue.

Protonation of histidines and other titratable residues is assigned a pH of 7, based on the PDBFixer tool in OpenMM (OpenMM. [accessed 2021 Oct 23]. https://openmm.org/ - GitHub—openmm/pdbfixer: PDBFixer fixes problems in PDB files. [accessed 2021 Nov 27]. https://github.com/openmm/pdbfixer).

This approach has been previously described in detail [187] and the main concepts and quantities constituting the model can be summarized as follows:

- **Electron density:** The distribution of electrons in a given molecular system. The electron density determines the nature and strength of the chemical bonds between interacting molecules. Such an "electron cloud" is the main emerging property of the underlying atomic structure in defining the chemical characteristics of a molecule.

- **Fragment:** The modular elements into which the electron cloud can be partitioned, for example, an amino acid. The model partitions the electron cloud into physically consistent regions and/or verifies the consistency of a pre-defined partitioning; every such region is defined as a fragment.

- **FBO:** The descriptor of the inter-fragment interactions. FBO is the main quantity used in the model to represent the connection pattern of the fragments of interacting molecules.

- **Fragment interactions:** From the results of the model and the features of the fragments it is then possible to calculate the interaction strength between any two fragments. Such interaction has both a chemical/short-range term that is always attractive, and an electrostatic/long-range term that can be attractive or repulsive.

- **Final output:** At the end of the simulation, BigDFT provides a simple representation of the strength of interaction between fragments of the two molecules. The model can describe the energy and nature of the acting chemical bonds. This enables a mechanistic explanation and/or prediction of how a specific amino acid impacts the interactions with Alpha-amylase and vice-versa.

- **Hardware requirements:** The model requires massively parallel calculations via high performance computing. On a modern supercomputer, hundreds of simulations can be performed in a time frame of one hour.

2.2 Results and discussion

Based on the growing need to find new plant-based bioactive compound useful in the treatment of obesity and diabetes, 10 *P. vulgaris* cultivars have been tested for their inhibitory activities against digestive enzymes and for the presence of anti-nutritional factors.

Many enzymes are involved in the carbohydrate digestion. Before to be absorbed in the last intestinal tract, starch is hydrolyzed by a combination of salivary and pancreatic α-amylase and α-glucosidase [195]. Their role in the increasing of post prandial glucose can be reduced by a series of molecules including the protein α-AI. Various studies indicate the positive role of α-AI extract from *P. vulgaris* in antidiabetic and anti-obesity treatments [166].

In order to assess the inhibitory activity of the cultivar's extracts, it is first necessary to identify a method that allows to record the activity of these enzymes and then quantify the inhibitory activity.

Instead, the presence of antinutritional factors, such as lectins, is an undesirable condition. It is therefore necessary to carry out a screening of all cultivars to choose the starting materials to produce new nutraceutical ingredients for the treatment of obesity and diabetes.

Moreover, since the sequences and structural features of α-AI proteins are important to better understand the details of the inhibition process, our research objective was to construct partial structural models for ten cultivars of *P. vulgaris* α-AI.

Using sequence and structure information available in gene databases, the aminoacidic sequences are partially reconstructed. Such models, together with mechanistic

characterization could help to gain insight into these inhibitors and their interactions with α-amylase enzyme.

2.2.1 Development of an assay to quantify α–amylase and α-glucosidase activity

Several methods employing different techniques have been used to assess α-amylase activity. In most of studies reviewed it was measured by quantifying the reducing sugar in accordance with Bernfeld assay [29,30,50,63,80,196]. Usually, an appropriate blank was prepared without α-amylase.

An extra control with α-amylase without the substrate starch has unfortunately evidenced an important background signal (Figure 2.2).
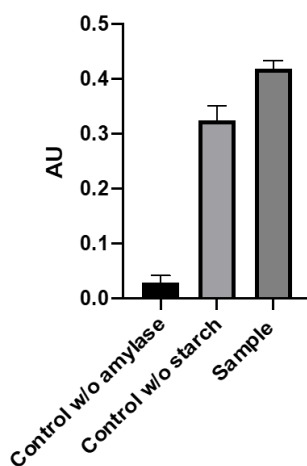
Figure 2.2: Graphical representation of α-amylase activity recorded with DNS assay.

This phenomenon is correlated to the contamination of commercial preparation of porcine pancreatic α-amylase (Type VI-B A3176-2.5MU-SIGMA) with lactose added as preservative. This finding suggests the need to perform a further blank control with amylase before proceeding to quantify its activity or to pay particular attention to the

composition of the used enzyme. However, this blank presents a too high absorbance, affecting precision and sensibility of the measurements.

The commercial α-amylase was even dialyzed (data not shown), but this procedure was not effective, greatly affecting the stability of the enzyme.

Thus, the presence of lactose led to discard this assay even though this is one of the most widely used assay in literature.

Therefore the method chosen for the quantification of α-amylase activity was 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3) assay in accordance with literature [197,198].

In this synthetic substrate a maltotriose (G3) is substituted at the reducing end with a 2-chloro-4-nitrophenol. The enzyme hydrolyzes CNP-G3 to release 2-chloro-4-nitrophenol (CNP), maltotriose (G3), 2-chloro-4-nitrophenyl-α-D-maltoside (CNP-G2), and glucose (Figure 2.3). This suggests a multi-splitting site attack. The hydrolytic cleavage can occur at the bond both between G3 and aglycone CNP, and between CNP-G2 and G.

The formation of CNP can be monitored at 405 nm reflecting amylase activity. As previously suggested, potassium thiocyanate (KSCN) was added to increases the rates of CNP-release [177].

2-chloro-*p*-nitrophenyl-α-D-maltotrioside (**CNP-G3**)   maltotriose   2-chloro-*p*-nitrophenol (**CNP**)   glucose   2-chloro-4-nitrophenyl-α-D-maltoside (**CNP-G2**)
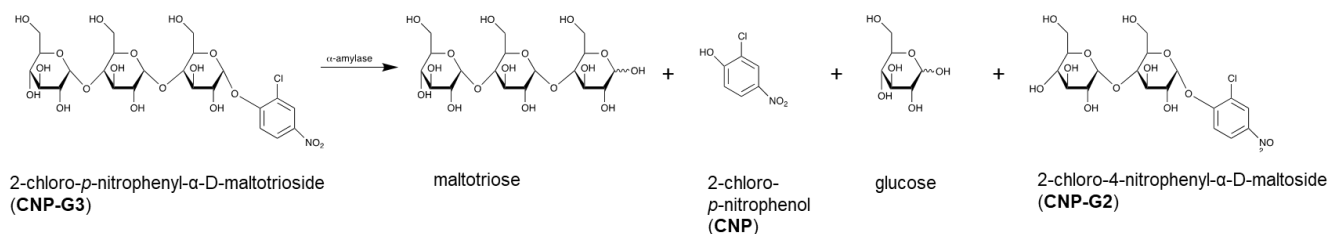
Figure 2.3 Reaction scheme of the hydrolysis of CNP-G3 by α-amylase enzyme

This spectrophotometric method is quite more expensive than DNS one, but it allows a negligible background interference (data not shown). To ensure stability and durability,

the amylase enzyme was suspended in 50% glycerol. As shown in Figure 2.4, this assay gave a great reproducibility and a large linear range up to 30'. To validate the assay, an extract from a food supplement based on common bead seeds was analysed as a positive control, resulting in 882 ± 43 IAU/g of dry extract.
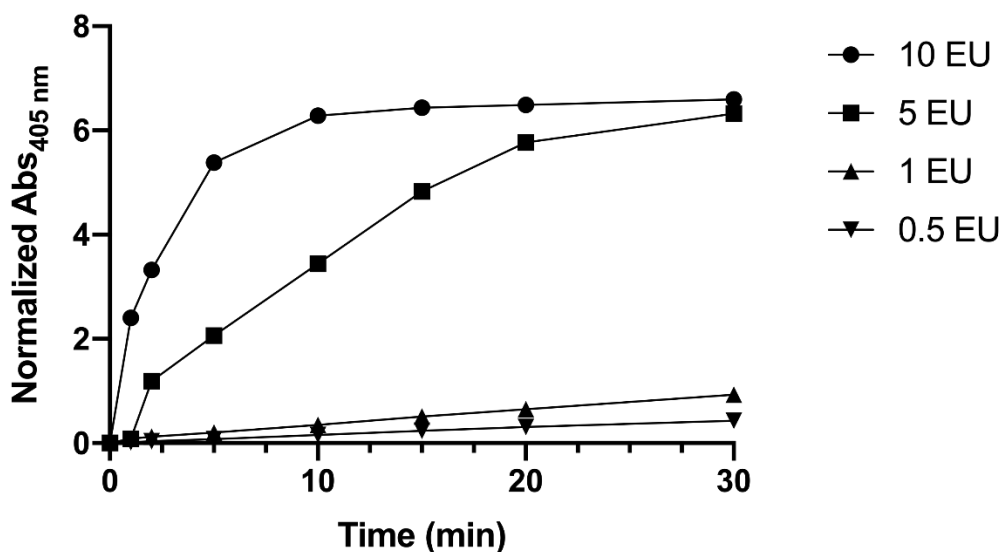
Figure 2.4: Time-dependent increase in absorbance at 405 nm during CNP hydrolysis by different amount of α-amylase.

To quantify α-glucosidase activity a similar synthetic substrate was used: *p*-nitrophenyl-α-D-glucopyranoside (pNPG). In this case, no incubation time was required before the substrate was added.

2.2.2 α−Amylase and α-glucosidase inhibitory activity: a comparison among cultivars

Considering the role of α-amylase and α-glucosidase enzymes in sugar absorption, the presence of α- amylase and α-glucosidase inhibitory activities is a desirable condition in a vegetable extract.

The comparison of raw extract of the examined cultivars indicated that eight out of ten inhibit α-amylase porcine pancreatic enzyme, as shown in Figure 2.5 a). Three varieties, Fazadu Nieddu, Nieddone and Bianco have the highest inhibitory activity (respectively 351±19, 356±26, 376±12 IAU/g) while two cultivars Fasolu Piantau and Faitta a Cavanedda have not α-AI inhibitor activity (Table 2.3). These last ones have not inhibitory activity also on human salivary α-amylase, as shown in Figure 2.5 b). Instead, the highest inhibitory power against human salivary were recorded in Fasolu, Faitta Sorgonese and Fazadu Nieddu (respectively 196 ± 5, 185 ± 30, 170 ± 3IAU/g) (Table 2.3).

The lack of inhibitory activity in Faitta a Cavanedda and Fasolu Piantau resulting in not suitable starting material to produce commercial supplements.

The α-glucosidase inhibitor activity was recorded in all cultivars. (Figure 2.6). Fasolu, Fazadu Nieddu and Bianco have the highest amount of inhibitory activity (respectively 182 ± 21, 118 ± 19, 108 ± 18 IGU/g) with significant differences reported.
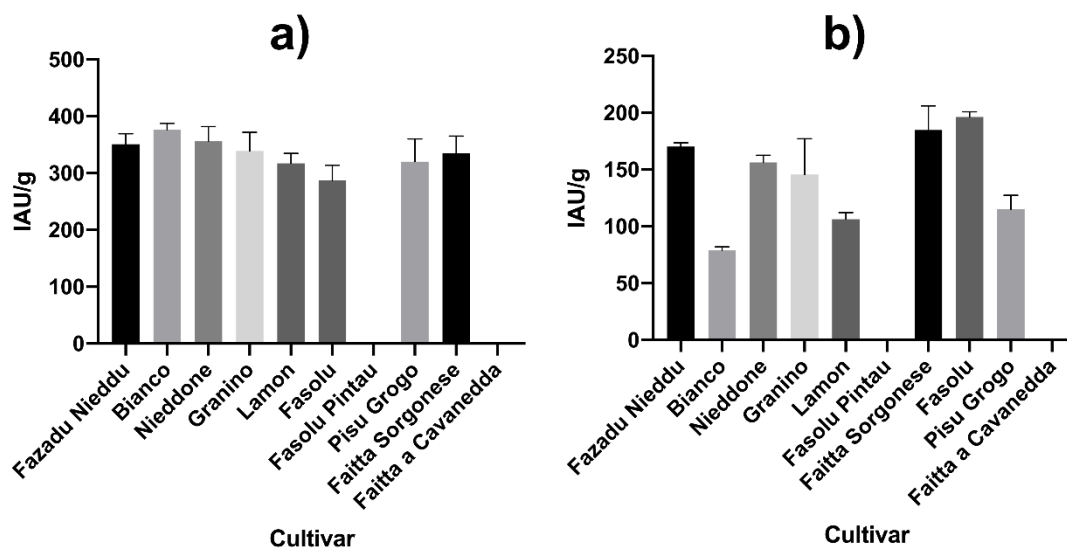
Figure 2.5: α-amylase inhibitory activity of the 10 cultivars raw extract on porcine amylase (2.4a) and on human amylase (2.4b).
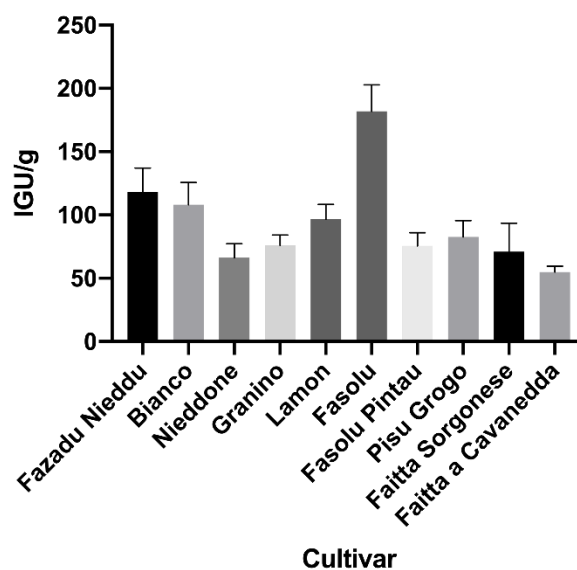
Figure 2.6: α-glucosidase inhibitory activity of the 10 cultivars raw extract on S. cerevisiae glucosidase.

No significant correlation has been observed between the inhibitory activity of porcine α-amylase and α-glucosidase (p>0.05; r=0.31). This aspect is confirmed by the presence of α-glucosidase inhibitory activity in the two cultivars without α-AI. As previously suggested different classes of compounds could be involved in the inactivation of α-AI/α-glucosidase inhibitors [32].

The red colour prevails in cultivars with the low inhibitory activity against porcine and human α-amylase (Lamon and Pisu Grogu) and in the cultivars without inhibitory activity (Fasolu Pintau and Faitta a Cavanedda), but the lowest values were recorded in light-coloured cultivar (Fasolu against porcine pancreatic α-amylase and Bianco against human α-amylase) in accordance with what previously observed [32].

Otherwise, in our work no evident correlation with the seed colour and the inhibitory activity can be observed: in fact, for instance the light-coloured cultivar Bianco showed low inhibitory activity against human α-amylase but high activity against porcine pancreatic α-amylase.

On the contrary Fasolu, that had the lowest inhibitory activity against porcine α-amylase shows the highest activity against human α-amylase. The other cultivar with high inhibitory activity against porcine α-amylase (Nieddone) is black coloured.

Cultivar	Porcine amylase	Human amylase	S. cerevisiae glucosidase	Total proteins
	IAU/g		IGU/g	(mg/g)
Fazadu Nieddu	351 ± 19^a	170 ± 3^a	$118 \pm 19^{a,b}$	25.8 ± 1.6^a
Bianco	376 ± 12^a	79 ± 6^c	$108 \pm 18^{a,b}$	35.4 ± 1.7^a
Nieddone	356 ± 26^a	$156 \pm 6^{a,b}$	66 ± 11^b	33.3 ± 3.8^a
Granino	339 ± 33^a	$146 \pm 31^{a,b}$	76 ± 8^b	24.2 ± 2.4^a
Lamon	317 ± 17^a	$106 \pm 6^{b,c}$	$97 \pm 12^{a,b}$	26.7 ± 1.0^a
Fasolu	287 ± 27^a	196 ± 5^a	182 ± 21^a	22.5 ± 3.0^a
Fasolu Pintau	n.d.	n.d.	$76 \pm 10^{a,b}$	21.9 ± 2.6^a
Pisu Grogu	320 ± 40^a	$115 \pm 12^{b,c}$	$83 \pm 13^{a,b}$	23.2 ± 2.9^a
Faitta Sorgonese	335 ± 30^a	185 ± 30^a	$71 \pm 22^{a,b}$	24.6 ± 2.9^a
Faitta a Cavanedda	n.d.	n.d.	55 ± 5^b	31.0 ± 5.4^a

Table 2.3: Inhibitory activity of examined extract on α-amylase, α-glucosidase, and the total protein content of raw extracts. Data are expressed as mean ± SEM (n =5). Mean values for the same analysis having different letters are significantly different (p<0.05; One–way ANOVA followed by the Bonferroni Multiple Comparisons Test). n.d. not detectable.

On the other hand, the other cultivars with great inhibitory activity against human α-amylase (Faitta Sorgonese and Fazadu Nieddu) are white and spot white/black coloured.

More investigation are anyway needed to verify if the colour of the outer seed tegument influenced the inhibitory activity, but an earlier study seems to confirm the absence of correlation [148]

The content of total proteins ranges from 21.9 ± 2.6 mg/g in Fasolu Pintau to 35.4 ± 1.7 mg/g in Bianco (Table 2.3). This variability could be related to genetic diversity or different agro-tecniques and different *P. vulgaris* growing conditions.

No correlation was found between the total content of protein and the inhibition of α-amylases and α-glucosidase ($p > 0.05$, $0.04 < r < 0.16$) in accordance with what observed with other bean cultivars [32]. But it is interesting that without Faitta a Cavanedda and Fasolu Pintau zero values, a correlation between total protein and porcine α-amylase inhibition occurs ($p < 0.05$, $r = 0.79$, Figure 2.7). The other p values remained greatly above 0.05.
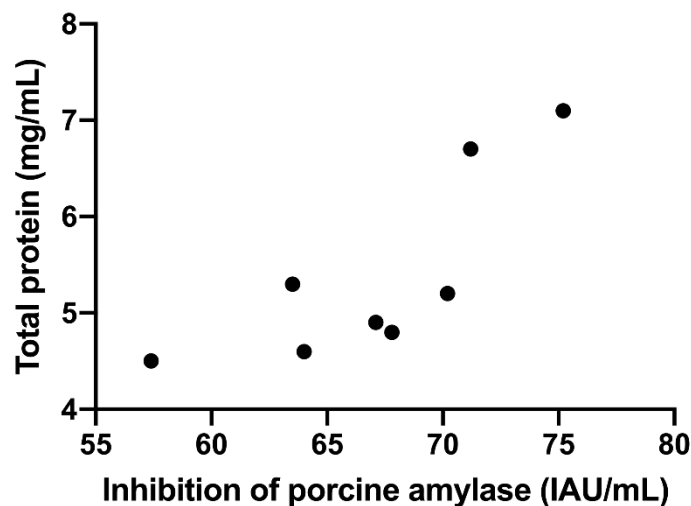
Figure 2.7 Correlation between total protein content and α-AI activity without zero values (p < 0.05, r = 0.79)

The presence of α-amylase inhibition activity has been reported in different common bean varieties: Pinto cv [199], white bean [200], and red kidney bean [201], but up to now no

studies had been yet conducted on the inhibitory power of extracts of theses Sardinian varieties. Other Mediterranean and southern Italian common bean cultivars have been studied for their action on digestive enzymes and for the role of polyphenol content in the inhibition of α-amylase and α-glucosidase [32,202].

The content of polyphenols and their inhibitory power on α-amylase and α-glucosidase was investigated also on Mexican and Brazilian cultivar, on cranberry beans, and on black turtle beans.[45,203,204].

On the contrary the leaves extract from Chilean bean landraces has only α-glucosidase inhibitory [205]. High variability in α-AI activity was recorded also for Indian common bean cultivar [206], in a sort of agreement with our data.

In this respect, the study of polyphenol content in Sardinian beans will be useful for a better characterisation of Sardinian cultivars (even if the focus of the present study has been limited to activity from protein inhibitors).

2.2.3 Hemagglutination activity

Hemagglutinins belong to the lectin family, a heterogeneous group of glycoproteins resistant to the intestinal proteolysis that agglutinated blood cells [207]. These proteins are contained in raw beans but their activity can be eliminated by heat temperature [141]. Since common beans are usually consumed after cooking no side effects occur after their ingestion.

However, it is necessary to pay particular attention on the residual lectins activity present in food supplements [208]. In this respect, there is a clear interest to identify cultivars with low hemagglutination power.

With this aim, human red blood cells agglutination has been performed with serial dilutions of our extracts, identifying the minimum dilution able to still agglutinate cells (MAC).

Previous studies have shown that lectins from the family *Fabaceae* usually cause agglutination. Three plant species, *Pisum sativum*, *Phaseolus vulgaris*, and *Glycine max,* caused agglutination of erythrocytes from all blood groups [206].

All screened cultivars tested in this study had hemagglutinin activity even with significant dilution (average value 12.5 mg/mL). Only Nieddone showed no activity even at the highest concentration tested, 200 mg/mL (Table 2.4) and Figure 2.8.

Cultivar	MAC (mg/mL)
Fazadu Nieddu	12.5
Bianco	6.25
Nieddone	>200
Granino	12.5
Lamon	12.5
Fasolu	25
Fasolu Pintau	6.25
Pisu Grogu	12.5
Faitta Sorgonese	12.5
Faitta a Cavanedda	12.5

Table 2.4: Hemagglutination activity recorded in 10 common bean cultivars Results are expressed as the Minimum protein Concentration able to Agglutinate sample (MAC, mg/mL).
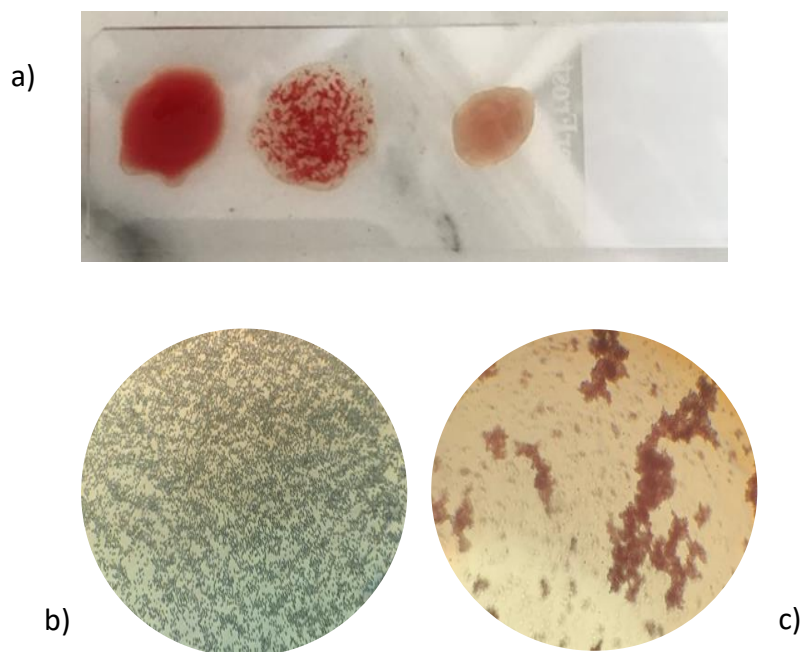
Fig. 2.8: a) Different agglutination degree; b) Negative control (no agglutination); c) several large aggregates (Lamon cv).

This desirable condition is reported also for other *P. vulgaris* cultivars: Great Northern [32] Tapiramo and Mottled bean [209].

Other seeds from members of *Fabaceae* family have no hemagglutination activity such as *Arachis hypogea* [210]. Particular attention must be paid to the case of *Glycine max.* In fact, the hemagglutination activity was recorded in soybean by some authors [209,210], but not by others [32]. This discrepancy could be related to the different soybean cultivar investigated.

Typically only white common beans extract are used for commercial supplements [38,211] and previous study have found that the coloured common beans had the highest hemagglutination power [81]. However, the data here reported show that also black coloured beans may have not this antinutritional factors.

The absence of hemagglutination power, the presence of α-AI/α-glucosidase inhibitors makes Nieddone the most interesting cultivar as starting material to produce food

supplements or commercial product (snack or cream) lectin-free in accordance with what suggested by other studies [212].

2.3 Molecular results

The molecular results obtained, as well as contributing to the enlargement of the genetic information of this plant, put a new piece in the puzzle of the knowledge of the primary structure of α-AI protein, through the isolation, in all ten cultivars analysed, of the transcript coding for the β-subunit of the protein.

2.3.1 PCR analysis of cDNA obtained from *P. vulgaris* cultivars

Exploiting the high amino acid sequence homology among α-AIs proteins of the genus *Phaseolus*, we designed partially degenerate primer pairs using the CODEHOP bioinformatics program. Using the pairs of primers available on cDNAs, we obtained sequenceable fragment which allowed us to reconstruct part of the nucleotide sequence of α-AI gene of each cultivar. We have reconstructed about 70% of the entire nucleotide sequence of the pv28-α-AI cultivar and about 30% of the other 9 cultivars. The nucleotide sequences obtained, compared with those deposited in the NCBI GenBank and in the EMBL-EBI database (https://www.ebi.ac.uk/), showed a high degree of similarity with several plant α-AIs sequences. The greatest degree of similarity emerged from the alignment with *P. vulgaris* α-AI-1 (accession No. CAD28835) and *P. vulgaris* α-AI-4 (accession No U84390.1) isoforms. Once in silico translation of the nucleotide sequence into amino acid, the primary structure obtained for each cultivar was aligned with the proteins deposited in the UniProt database (https://www.uniprot.org/uniprotkb/) obtaining, as result, a homology with the β-subunit of α-AI-1(accession No P02873.1)

and α-AI-4 (accession No AAB42070.1) of *P. vulgaris* (Figure 2.9).

As shown in Figure 2.9, PV28-α-AI was the protein from which we deduced most of the information on the primary structure having obtained 152 amino acids. Its sequence aligns from amino acid 91 to amino acid 243 of P02873 α-AI-1 and from amino acid 89 to amino acid 241 of AAB42070.1 α-AI-4 isoform (the numbering refers to the preprotein). As regards the other nine proteins we obtained 77 amino acids of the whole sequence of α-AI. Eight of the ten sequences (i.e. all with the exception of PV20-α-AI and PV152-α-AI), align from amino acid 129 to amino acid 205 of P02873 α-AI-1 protein and from amino acid 127 to amino acid 203 of AAB42070.1 α-AI-4 isoform. Specifically, for the proteins PV177-α-AI, PV147-α-AI, PV124-α-AI, PV121-α-AI, PV113-α-AI, PV1-α-AI and PV-LAM-α-AI we obtained primary structures that showed very high sequence identity compared with β subunit of α-AI-1 (94%) and α-AI-4 (89%) of *P. vulgaris* isoforms (Figures 2.10 A and B), while for PV152-α-AI and PV20-α-AI the highest homology value was less than 50%.

```
                                          [___ signal peptide____]✂[_____ α subunit_____

P02873.1-alpha-AI-1              MIMASSKLLSLALFLALLSHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSYNSY    60
AAB42070.1-alpha-AI-4           --MASSNLLSLALFLVLLTHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSHNSY    58
Granino-ACC28-(OP329712)        ----------------------------------------------------------     0
Pisu-Grogu-ACC152-(OP441068)    ----------------------------------------------------------     0
Fasolu-ACC20-(OP441067)         ----------------------------------------------------------     0
Bianco-ACC124-(OP441071)        ----------------------------------------------------------     0
Lamon-(OP441072)                ----------------------------------------------------------     0
Fazzadu-a-Nieddu-ACC121-(OP441070) -------------------------------------------------------     0
Nieddone-ACC177-(OP530245)      ----------------------------------------------------------     0
Fasolu-Pintau-ACC113-(OP441066) ----------------------------------------------------------     0
Faitta-Sorgonese-ACC1-(OP779318) ---------------------------------------------------------     0
Faitta-a-Cavanedda-ACC147-(OP441069) -----------------------------------------------------     0

                                                                      ]✂[___  β subunit_____

P02873.1-alpha-AI-1             DSMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPVQPESKGDT   120
AAB42070.1-alpha-AI-4           DSMSRAFYSAPIQIRDSTTGNVASFDSNFTMNIRTHRQANSAVGLDFVLVPVQPESKGDT   118
Granino-ACC28-(OP329712)        ----------------------------LIFRTHRQANSAVGLDFVLVPVQPESKGDT    30
Pisu-Grogu-ACC152-(OP441068)    ----------------------------------------------------------     0
Fasolu-ACC20-(OP441067)         ----------------------------------------------------------     0
Bianco-ACC124-(OP441071)        ----------------------------------------------------------     0
Lamon-(OP441072)                ----------------------------------------------------------     0
Fazzadu-a-Nieddu-ACC121-(OP441070) -------------------------------------------------------     0
Nieddone-ACC177-(OP530245)      ----------------------------------------------------------     0
Fasolu-Pintau-ACC113-(OP441066) ----------------------------------------------------------     0
Faitta-Sorgonese-ACC1-(OP779318) ---------------------------------------------------------     0
Faitta-a-Cavanedda-ACC147-(OP441069) -----------------------------------------------------     0


P02873.1-alpha-AI-1             VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK   180
AAB42070.1-alpha-AI-4           VTVEFDTFLSRVSIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTER   178
Granino-ACC28-(OP329712)        VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    90
Pisu-Grogu-ACC152-(OP441068)    --------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITPPRRSSRFLCQTLLRER    52
Fasolu-ACC20-(OP441067)         --------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITPPRRSSRFLCQTLLRER    52
Bianco-ACC124-(OP441071)        --------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK    52
Lamon-(OP441072)                --------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    52
Fazzadu-a-Nieddu-ACC121-(OP441070) -----LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    52
Nieddone-ACC177-(OP530245)      --------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK    52
Fasolu-Pintau-ACC113-(OP441066) --------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    52
Faitta-Sorgonese-ACC1-(OP779318) -------LSRISIDVNNNDIKSRAWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    52
Faitta-a-Cavanedda-ACC147-(OP441069) ---LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    52
                                        ***:**********  *:     .:. ..   .  :    .   .   .  :

P02873.1-alpha-AI-1             SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI   240
AAB42070.1-alpha-AI-4           ATTSLPQWRLEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNV   238
Granino-ACC28-(OP329712)        SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI   150
Pisu-Grogu-ACC152-(OP441068)    ATTSLPQWSWRKKCMT-------------------GCPWASPPP--------------    77
Fasolu-ACC20-(OP441067)         ATTSLPQWSWRKKYMT-------------------GCPWASPPP--------------    77
Bianco-ACC124-(OP441071)        SNDVSTTVELEKEVYDWVSVGFSAT---------------------------------    77
Lamon-(OP441072)                SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------    77
Fazzadu-a-Nieddu-ACC121-(OP441070) SNNVSTTVELEKEVYDWVSVGFSAT------------------------------    77
Nieddone-ACC177-(OP530245)      SNDVSTTVELEKEVYDWVSVGFSAT---------------------------------    77
Fasolu-Pintau-ACC113-(OP441066) SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------    77
Faitta-Sorgonese-ACC1-(OP779318) SNNVSTTVELEKEVYDWVSVGFSAT--------------------------------    77
Faitta-a-Cavanedda-ACC147-(OP441069) SNNVSTTVELEKEVYDWVSVGFSAT----------------------------    77
                                :.          .*:

P02873.1-alpha-AI-1             VLNKIL     246
AAB42070.1-alpha-AI-4           VLNQIL     244
Granino-ACC28-(OP329712)        VL----     152
Pisu-Grogu-ACC152-(OP441068)    ------      77
Fasolu-ACC20-(OP441067)         ------      77
Bianco-ACC124-(OP441071)        ------      77
Lamon-(OP441072)                ------      77
Fazzadu-a-Nieddu-ACC121-(OP441070) ------   77
Nieddone-ACC177-(OP530245)      ------      77
Fasolu-Pintau-ACC113-(OP441066) ------      77
Faitta-Sorgonese-ACC1-(OP779318) ------     77
Faitta-a-Cavanedda-ACC147-(OP441069) ------ 77
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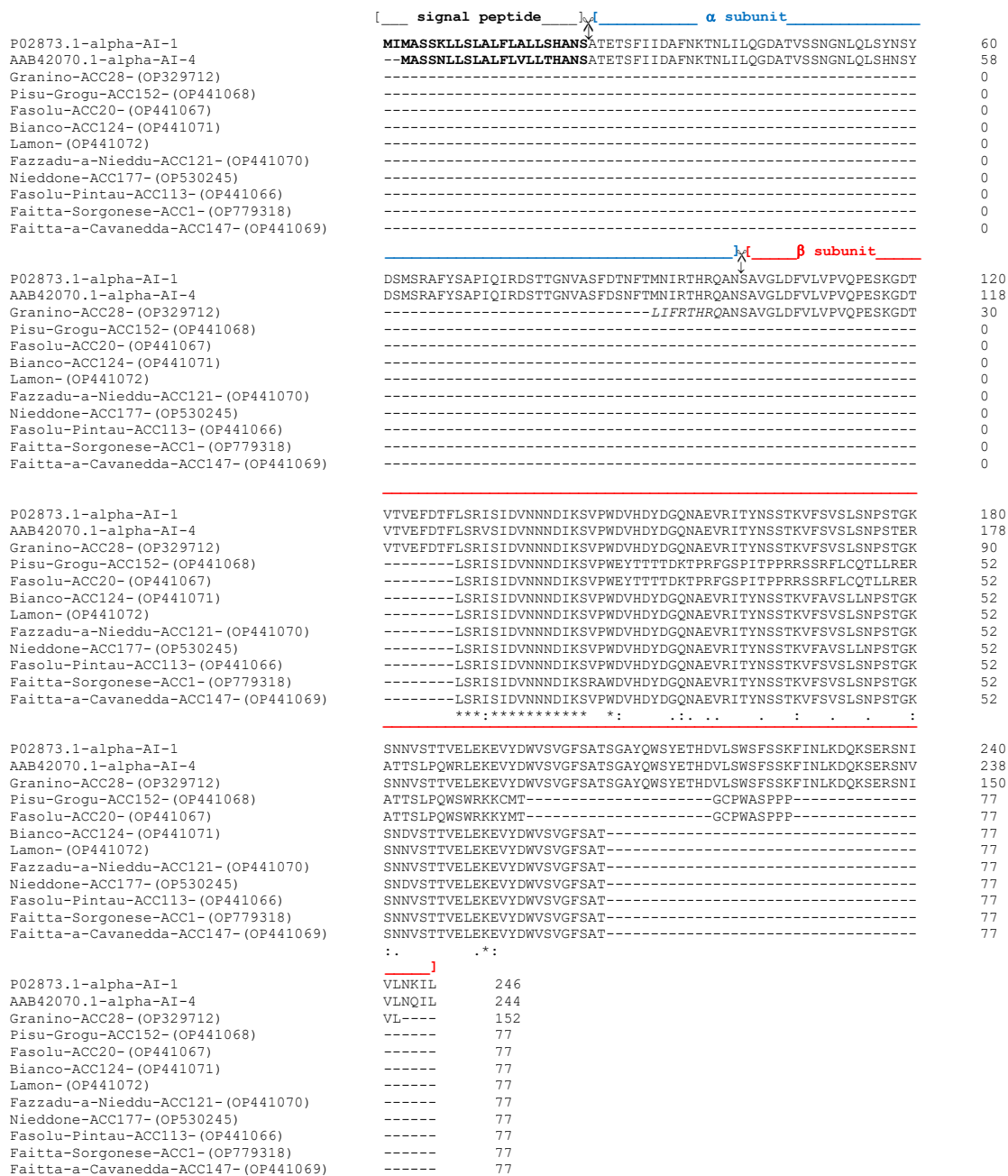
Figure 2.9: Alignment of α-AIs cultivars amino acid sequences with homologous α -AIs sequences from GenBank. Numbers on the left indicate the position of the amino acids in each protein. An asterisk (*) denotes identical residues; double dots (:) represent a conserved residue substitution; a single dot (.) shows partial conservation of the residue. The figure shows the proteolytic cuts (symbol ✂) that the preprotein undergoes to give rise to the α and β subunits

A

```
P02873.1-alpha-AI-1                          VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK        180
Granino-ACC28-(OP329712)                     -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         90
Bianco-ACC124-(OP441071)                     -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK         52
Lamon-(OP441072)                             -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         52
Fazzadu-a-Nieddu-ACC121-(OP441070)           -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         52
Nieddone-ACC177-(OP530245)                   -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK         52
Fasolu-Pintau-ACC113-(OP441066)              -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         52
Faitta-Sorgonese-ACC1-(OP779318)             -------LSRISIDVNNNDIKSRAWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         52
Faitta-a-Cavanedda-ACC147-(OP441069)         -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK         53
                                                    *************** ************************:*** ******

P02873.1-alpha-AI-1                          SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI       240
Granino-ACC28-(OP329712)                     SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI       150
Bianco-ACC124-(OP441071)                     SNDVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Lamon-(OP441072)                             SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Fazzadu-a-Nieddu-ACC121-(OP441070)           SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Nieddone-ACC177-(OP530245)                   SNDVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Fasolu-Pintau-ACC113-(OP441066)              SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Faitta-Sorgonese-ACC1-(OP779318)             SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Faitta-a-Cavanedda-ACC147-(OP441069)         SNNVSTTVELEKEVYDWVSVGFSAT---------------------------------        78
                                             **:*********************
```

B

```
AAB42070.1-alpha-AI-4                        VEFDTFLSRVSIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTERAT        180
Granino-ACC28-(OP329712)                     ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        92
Bianco-ACC124-(OP441071)                     ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGKSN        54
Lamon-(OP441072)                             ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        54
Fazzadu-a-Nieddu-ACC121-(OP441070)           ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        54
Nieddone-ACC177-(OP530245)                   ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGKSN        54
Fasolu-Pintau-ACC113-(OP441066)              ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        54
Faitta-Sorgonese-ACC1-(OP779318)             ------LSRISIDVNNNDIKSRAWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        54
Faitta-a-Cavanedda-ACC147-(OP441069)         ------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSN        55
                                                  ***:**********  ***********************:*** ****  ::.

AAB42070.1-alpha-AI-4                        TSLPQWRLEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNVVL        240
Granino-ACC28-(OP329712)                     NVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNIVL        152
Bianco-ACC124-(OP441071)                     DVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Lamon-(OP441072)                             NVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Fazzadu-a-Nieddu-ACC121-(OP441070)           NVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Nieddone-ACC177-(OP530245)                   DVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Fasolu-Pintau-ACC113-(OP441066)              NVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Faitta-Sorgonese-ACC1-(OP779318)             NVSTTVELEKEVYDWVSVGFSAT---------------------------------        77
Faitta-a-Cavanedda-ACC147-(OP441069)         NVSTTVELEKEVYDWVSVGFSAT---------------------------------        78
                                               .***************
```

Figure 2.10: (A) Alignment of eight α-AIs cultivars amino acid sequences with -AI-1 sequence (accession No P022873.1) of P. vulgaris. Numbers on the left indicate the position of the amino acids in each protein. An asterisk (*) denotes identical residues (94%); double dots (:) represent a conserved residue substitution; a single dot (.) shows partial conservation of the residue. (B) Alignment of the same sequences with α-AI-4 (accession No AAB42070.1) of P. vulgaris. The identical residues value is 89%. Alignments (A) and (B) show the last 77 of 152 amino acids obtained for the PV28-α-AI the sequence.

To better highlight the variations existing in the primary structure of PV152-α-AI and PV20-α-AI, we performed the alignment of their amino acid sequences with the primary structure of α-AI-1 and α-AI-4 isoforms. The alignment with the α-AI-1 isoform showed the presence of two gaps of 11 amino acids each extending from amino acid 161-172 and 189-200, and a percentage of identity of about 33% and (Figure 2.11A). Moreover, we observed that PV152-α-AI and PV20-α-AI primary structures share more similarity with the isoform α-AI-4. In fact, aligning their amino acids it is possible to observe a

percentage of homology of about 40% with the isoform α-AI-4 and the presence of only

one gap along the sequence (Figure 2.11 B)

A

```
P02873.1-alpha-AI-1            VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK   180
Pisu-Grogu-ACC152-(OP441068)   --------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFG-----------SPITPPRRSS   41
Fasolu-ACC20-(OP441067)        --------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFG-----------SPITPPRRSS   41
                                       ******************:      .:. ..     ↑ gap    :: *  ..

P02873.1-alpha-AI-1            SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETH--DVLSWSFSSKFINLKDQKSERS   238
Pisu-Grogu-ACC152-(OP441068)   -RFLCQTLL-----------RERATTSLPQWSWRKKCMTGCPWASPPP------------   77
Fasolu-ACC20-(OP441067)        -RFLCQTLL-----------RERATTSLPQWSWRKKYMTGCPWASPPP------------   77
                                . :. *:         ↑ gap    **:.  ***:..:       *:
```

B

```
AAB42070.1-alpha-AI-4          VEFDTFLSRVSIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTERAT   180
Pisu-Grogu-ACC152-(OP441068)   ------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITPPRRSSRFLCQTLLRERAT   54
Fasolu-ACC20-(OP441067)        ------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITPPRRSSRFLCQTLLRERAT   54
                                     ***:************:      .:. ..           ****

AAB42070.1-alpha-AI-4          TSLPQWRLEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNVVL   240
Pisu-Grogu-ACC152-(OP441068)   TSLPQWSWRKKCMTGCP-----------WASPPP-------------------------   77
Fasolu-ACC20-(OP441067)        TSLPQWSWRKKYMTGCP-----------WASPPP-------------------------   77
                               ******  .*:           ↑ gap  *:
```

Figure 2.11. (A) Alignment of amino acid sequences of PV152-α-AI and PV20-α-AI with α-AI-1 isoform of P. vulgaris. Numbers on the left indicate the position of the amino acids in each protein. An asterisk (*) denotes identical residues (33%); double dots (:) represent a conserved residue substitution; a single dot (.) shows partial conservation of the residue. The gaps respect the alignment with α-AI-1 isoform are shown with arrow ↑). (B) Alignment of the same sequences with α-AI-4 of P. vulgaris. The identical residues value is 40%.

2.3.2 Amino acid pattern and profile search results.

The α-AIs of *P. vulgaris* generally show small differences in their primary structures and

in their *N*-glycosylation sites. α-AI-1 was the first isoform of *P. vulgaris* to be isolated

and molecularly characterized [34,59]. In the endoplasmic reticulum, the preprotein

undergoes cleavage of the signal peptide and *N*-glycosylated sites on residues of Asn35,

Asn88, and Asn163 (the numbers refer to the positions of the residues in the preprotein).

The prediction of glycosylation sites was also obtained for other *P. vulgaris* inhibitor

isoforms, confirming what was previously obtained for α-AI-1 [53]. In this study, the

search for potential glycosylation features was conducted using *NetNGlyc* tool.

Considering the partial sequences obtained for all cultivars, our results are consistent with the finding that at least one potential *N*-glycosylation site occur, at the same amino acid position of α-AI-1, in eight of the deduced cultivars sequences. Instead, no potential site was found in PV152-α-AI and PV20-α-AI sequences (Figure 2.12). In addition to the potential *N*-glycosylation sites, the protein kinase C phosphorylation sites, were found in eight of the deduced sequences with NetPhos-3.1 tool. In PV152-α-AI and PV20-α-AI, in which no potential site for protein kinase C was found, a potential phosphorylation site (PRRSS) for the PKA is instead present (Figure 2.12).

We also investigated for the amino acid residues important for the inhibitor activity, obtaining evidence only for the PV28-α-AI protein of which we almost completely determined the sequence of the β subunit. The amino acid residues Y209, S212 and Y213 of β subunit (the numbering of the amino acids refers to the preprotein), important for the interaction with the α-amylase enzyme are conserved in PV28-α-AI protein (Figure 2.12).

```
P02873.1-alpha-AI-1                      MIMASSKLLSLALFLALLSHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSYNSY     60
AAB42070.1-alpha-AI-4                    --MASSNLLSLALFLVLLTHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSHNSY     58
Granino-ACC28-(OP329712)                 ------------------------------------------------------------      0
Pisu-Grogu-ACC152-(OP441068)             ------------------------------------------------------------      0
Fasolu-ACC20-(OP441067)                  ------------------------------------------------------------      0
Bianco-ACC124-(OP441071)                 ------------------------------------------------------------      0
Lamon-(OP441072)                         ------------------------------------------------------------      0
Fazzadu-a-Nieddu-ACC121-(OP441070)       ------------------------------------------------------------      0
Nieddone-ACC177-(OP530245)               ------------------------------------------------------------      0
Fasolu-Pintau-ACC113-(OP441066)          ------------------------------------------------------------      0
Faitta-Sorgonese-ACC1-(OP779318)         ------------------------------------------------------------      0
Faitta-a-Cavanedda-ACC147-(OP441069)     ------------------------------------------------------------      0


P02873.1-alpha-AI-1                      DSMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPVQPESKGDT    120
AAB42070.1-alpha-AI-4                    DSMSRAFYSAPIQIRDSTTGNVASFDSNFTMNIRTHRQANSAVGLDFVLVPVQPESKGDT    118
Granino-ACC28-(OP329712)                 ---------------------------LIFRTHRQANSAVGLDFVLVPVQPESKGDT     30
Pisu-Grogu-ACC152-(OP441068)             ------------------------------------------------------------      0
Fasolu-ACC20-(OP441067)                  ------------------------------------------------------------      0
Bianco-ACC124-(OP441071)                 ------------------------------------------------------------      0
Lamon-(OP441072)                         ------------------------------------------------------------      0
Fazzadu-a-Nieddu-ACC121-(OP441070)       ------------------------------------------------------------      0
Nieddone-ACC177-(OP530245)               ------------------------------------------------------------      0
Fasolu-Pintau-ACC113-(OP441066)          ------------------------------------------------------------      0
Faitta-Sorgonese-ACC1-(OP779318)         ------------------------------------------------------------      0
Faitta-a-Cavanedda-ACC147-(OP441069)     ------------------------------------------------------------      0


P02873.1-alpha-AI-1                      VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK    180
AAB42070.1-alpha-AI-4                    VTVEFDTFLSRVSIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTER    178
Granino-ACC28-(OP329712)                 VTVEFDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     90
Pisu-Grogu-ACC152-(OP441068)             -------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITFPRRSSRFLCQTLLRER     52
Fasolu-ACC20-(OP441067)                  -------LSRISIDVNNNDIKSVPWEYTTTTDKTPRFGSPITFPRRSSRFLCQTLLRER     52
Bianco-ACC124-(OP441071)                 -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK     52
Lamon-(OP441072)                         -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     52
Fazzadu-a-Nieddu-ACC121-(OP441070)       -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     52
Nieddone-ACC177-(OP530245)               -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFAVSLLNPSTGK     52
Fasolu-Pintau-ACC113-(OP441066)          -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     52
Faitta-Sorgonese-ACC1-(OP779318)         -------LSRISIDVNNNDIKSRAWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     52
Faitta-a-Cavanedda-ACC147-(OP441069)     -------LSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGK     52
                                                ***:**********  *:    .:. ..    .:   .  .   .  :

P02873.1-alpha-AI-1                      SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI    240
AAB42070.1-alpha-AI-4                    ATTSLPQWRLEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNV    238
Granino-ACC28-(OP329712)                 SNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNI    150
Pisu-Grogu-ACC152-(OP441068)             ATTSLPQWSWRKKCMT--------------------GCPWASPPP--------------     77
Fasolu-ACC20-(OP441067)                  ATTSLPQWSWRKKYMT--------------------GCPWASPPP--------------     77
Bianco-ACC124-(OP441071)                 SNDVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Lamon-(OP441072)                         SNNVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Fazzadu-a-Nieddu-ACC121-(OP441070)       SNNVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Nieddone-ACC177-(OP530245)               SNDVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Fasolu-Pintau-ACC113-(OP441066)          SNNVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Faitta-Sorgonese-ACC1-(OP779318)         SNNVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
Faitta-a-Cavanedda-ACC147-(OP441069)     SNNVSTTVELEKEVYDWVSVGFSAT-----------------------------------     77
                                         :.          .*:

P02873.1-alpha-AI-1                      VLNKIL    246
AAB42070.1-alpha-AI-4                    VLNQIL    244
AAB42071.1-alpha-AI-5                    VLNQIL    244
Granino-ACC28-(OP329712)                 VL----    152
Pisu-Grogu-ACC152-(OP441068)             ------     77
Fasolu-ACC20-(OP441067)                  ------     77
Bianco-ACC124-(OP441071)                 ------     77
Lamon-(OP441072)                         ------     77
Fazzadu-a-Nieddu-ACC121-(OP441070)       ------     77
Nieddone-ACC177-(OP530245)               ------     77
Fasolu-Pintau-ACC113-(OP441066)          ------     77
Faitta-Sorgonese-ACC1-(OP779318)         ------     77
Faitta-a-Cavanedda-ACC147-(OP441069)     ------     77
```

Figure 2.12. Alignment α-AIs sequences. Red boxes indicate N-glycosylation consensus sequences, black boxes indicate the protein kinase C phosphorylation sites. A potential PKA phosphorylation site was found in PV152-α-AI and PV20-α-AI sequences (blue box). The amino acid residues Y209, S212 and Y213 of β subunit, important for the interaction with the α-amylase enzyme are conserved in PV28-α-AI protein (highlighted in grey).

2.3.3 Deduced phylogenetic analysis and secondary structure

α-AI-1 (P02873.1) and α-AI-4 (AAB42070.1) sequences from *P. vulgaris* were used as out-group. Phylogenetic analysis confirmed a greater similarity of the sequences in PV152-α-AI and PV20-α-AI with isoform α-AI-4 respect to isoform α-AI-1 which is more closely related to the other 8 cultivars (Figure 2.13).
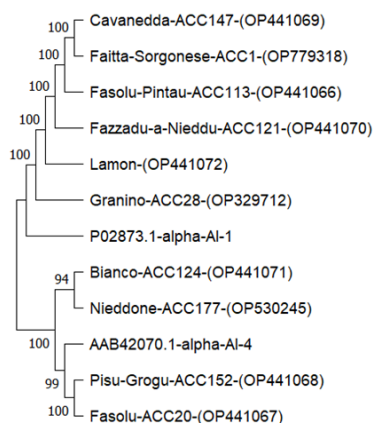

Figure 2.13: α-AI phylogenetic analysis. The phylogenetic tree was constructed using the Mega 11 tool. Values above branches indicate bootstrap percentages (500 replicates).
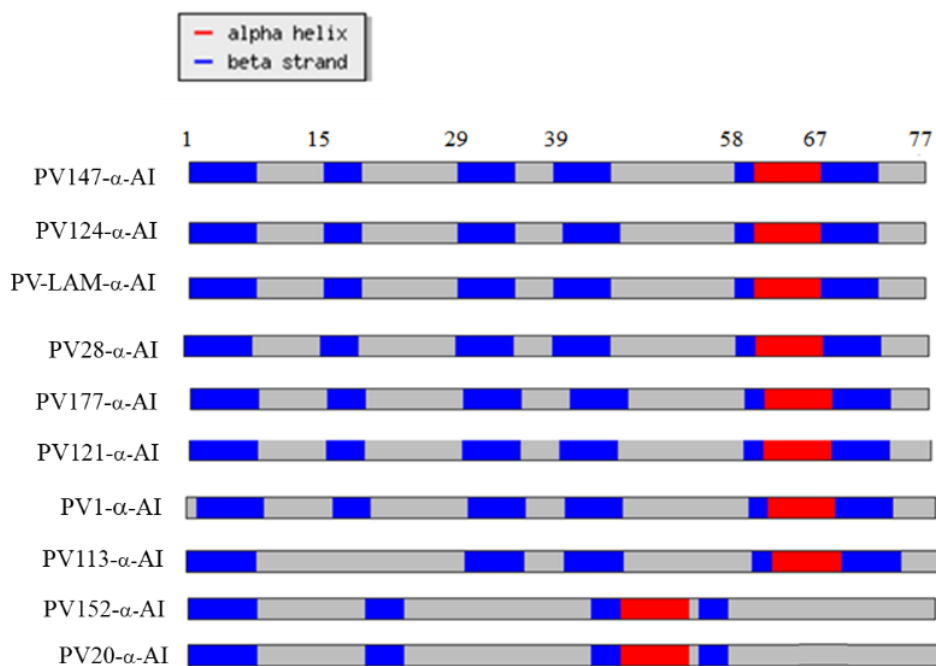
Figure 2.14: Chou-Fasman (http://cib.cf.ocha.ac.jp/bitool/MIX/) prediction of secondary structure of α-AIs region that includes 77 amino acid of β-subunit.

The *in-silico* reconstruction of the secondary structure showed differences in the distribution of the α-helices, β-sheets and coils of the PV152-α-AI and PV20-α-AI proteins if compared to the proteins deduced from the other cultivars (Figure 2.14).

Several factors could on the other explain the absence of inhibition towards α-amylase observed in the ACC 113 and ACC 147 cultivars. Since the entire primary structure of both proteins is not available, it is not possible to establish whether the amino acid residues important for protein maturation and activity are conserved [213]. Another explanation could be due to the presence in their cells of microRNAs (miRNAs) able to inhibit translation of α-AI. Usually, plant miRNAs are associated with Argonaute protein1 to promote RNA posttranscriptional gene silencing by coupling target sequences and determining RNA slicing and/or translation inhibition [214–216]. In the last few years, many miRNAs have been identified in *P. vulgaris* [217,218]. Within this second hypothesis, the presence of α-glucosidase inhibitory activity observed in the cultivars without α-AI could be linked to the presence of flavonol glycosides and caffeic acid derivatives in the homogenates. Recently, it has been demonstrated that a variety of *P. vulgaris* (chilean bean) extracts presented strong activity against α-glucosidase but were inactive towards α-amylase [205]. These hypotheses will be investigated by deepening the knowledge on the primary structure of α-AIs and investigating the possible presence of antisense miRNAs and phenolic substances active against α-glucosidase.

2.4 Mechanistic characterization of the interaction between α-amylase and α-AI

To understand if these structural differences highlighted by molecular analysis, could have influence on the protein/protein interaction between α-amylase and α-AI, we have also performed an *in silico* analysis. The contribution of each residue to the overall binding performance have been calculated, highlighting which amino acids facilitate or hinder binding, and how, using 1DHK PDB porcine pancreatic α-amylase enzyme and α-AI (P02873) aminoacidic sequence.

We used the FBO to identify the interface residues and chemical hotspot interactions.

Once the chemical connection among amino acids is identified, we assigned to each residue its contribution to the binding interaction between the two subsystems. We calculate these interaction terms from the output of the DFT code and interpret them as two parts. First, a long-range electrostatic attraction/repulsion term, defined from the electron distributions of each of the fragments (even when far apart, two fragments may still interact). The remaining term, which can only be attractive, is provided by the chemical binding between the fragments, and is nonzero only if the electronic clouds of the fragments superimpose (short-range). This term is correlated with the FBO strength, and we identified it as the chemical interaction.

By including long-range electrostatic terms, the decomposition enables us to single out relevant residues not necessarily residing at the interface. In this way, the model provides an *ab initio* representation of the RBD-ligand interactions as the final output.

Mechanistic characterization of the binding between α-amylase and its inhibitor from the bean *P. vulgaris* is shown in Figure 2.15. The data are plotted on the sequence of the bean. Letters represent single amino acid residues. "Å" is the distance of a residue to the nearest

atom of its ligand. "Tot" is the chemical/electrostatic force shown as attractive (blue) or repulsive (red), with darker colours indicating stronger effects.

The 'Tot' interaction is the sum of 'vdW', 'H', 'El' quantities, which corresponds respectively to the Van der Waals forces, chemical(steric) and electrostatic interactions and they are shown in separated lines above the 'Tot' line.

As we can notice, we can identify main two hotspots corresponding to aspartic acid D38 and to tyrosine Y186 (the numbering of the amino acids refers to the mature protein). The interaction is mainly governed by the chemical contribution by the closer amino acids. The values of the interaction are shown in the bottom panel of Figure 2.15.

Figure 2.16 shows the emerging interaction network that connects the relevant residues of the α-AI sequence from *P. vulgaris* (J01261) with the corresponding interface residues of the amylase. The network is drawn by following the FBO between amino acids of the respective sequence. Thicker lines indicate stronger chemical bonds. Nodes are coloured following the total interaction values, and their border is based on the FBO at the interface as per Figure 2.16.

In Figure 2.17 it is represented a highlight of the relevant part of the general interaction graph of Figure 2.16.

Unfortunately, the two hotspots (D38 and Y186) have not yet been sequenced by CODEHOP strategy in our cultivars. Only in the case of cultivar Granino (PV28-α-AI) it has been reconstructed an aminoacidic fragment corresponding to almost all β subunit, including Y186 position. This showed no differences from the aminoacidic reference sequence used for our analyses (PO28731).
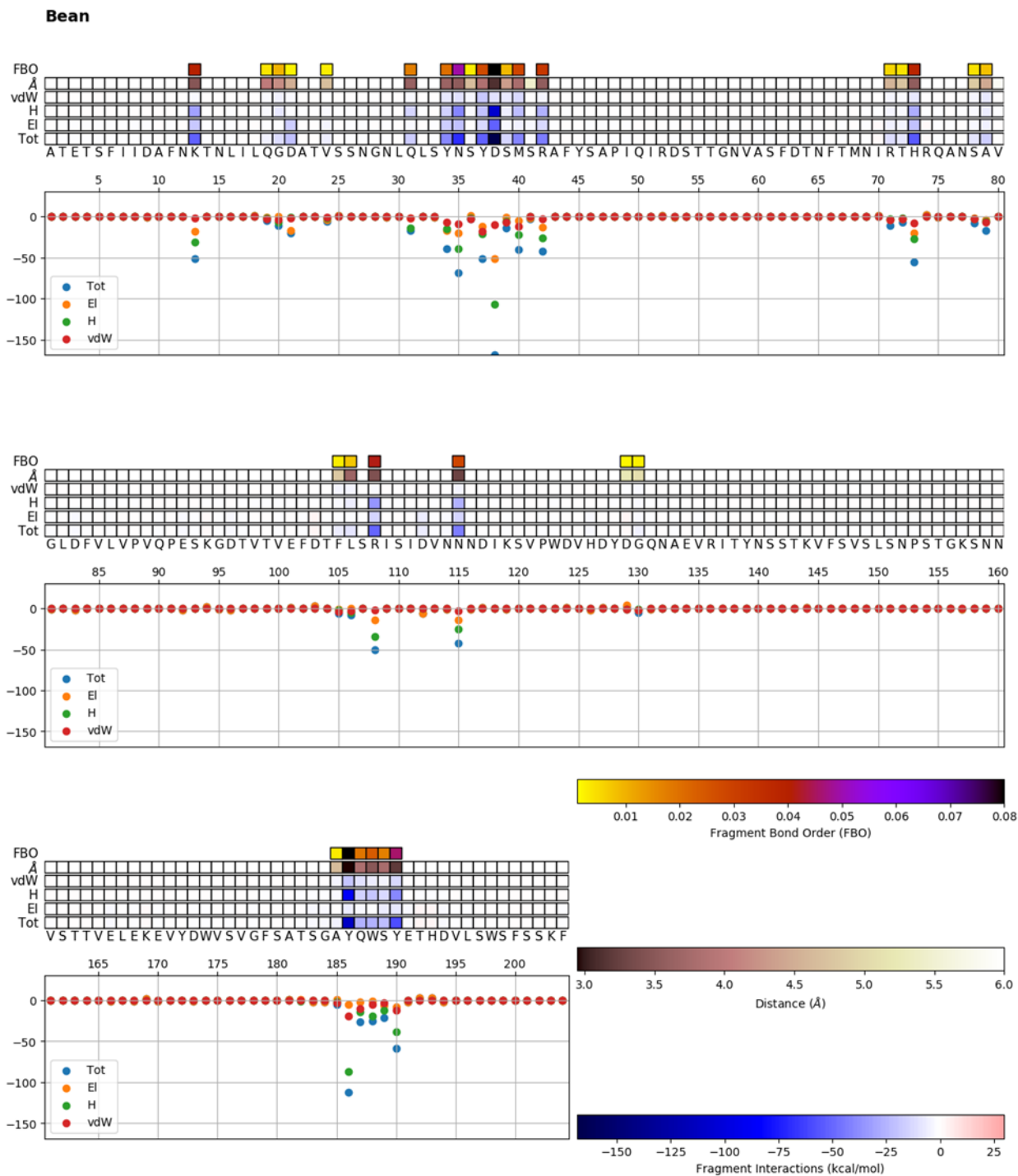
Figure 2.15: Mechanistic characterization of the binding between Alpha-amylase and his inhibitor from the bean Phaseolus vulgaris
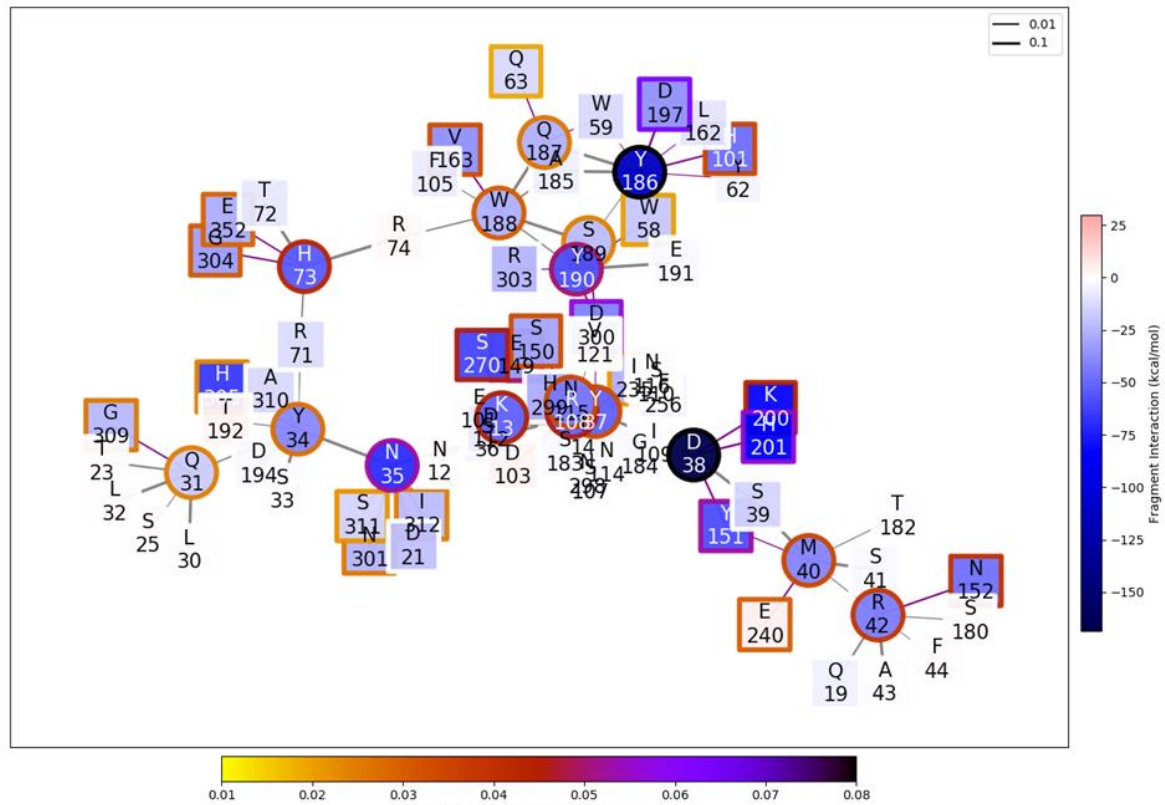
Figure 2.16: emerging interaction network that connects the relevant residues of α-AI from common bean with α-amylase
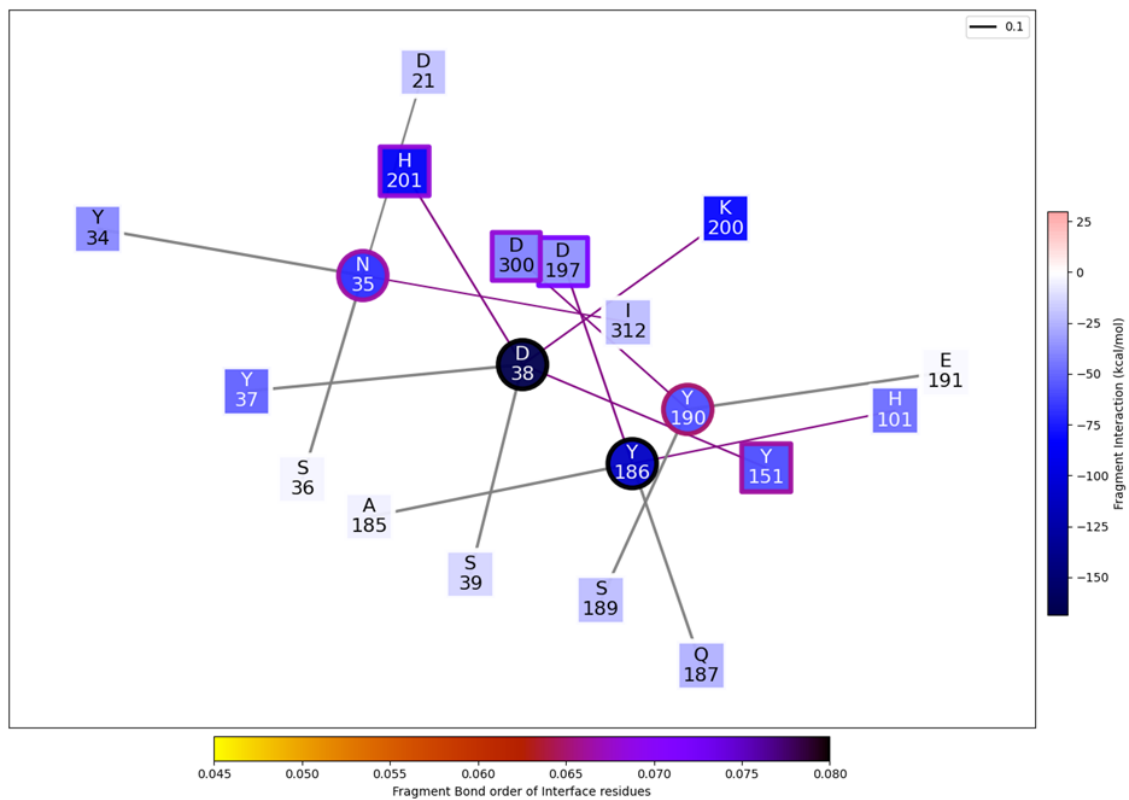
Figure 2.17: enlargement of relevant part of the general interaction graph

For the other cultivars, on the contrary, it is not yet possible to gain information about these hotspots. However, this can be an important incentive to complete the sequences from our samples, and thus get complete information about sequence/structure/activity relationships. Such pieces of knowledge can be in fact crucial to effectively screen unknown varieties or to develop new recombinant α-AI sequences with improved and modulated features.

2.5 Conclusions

The screening of 9 rare Sardinian common bean varieties and of one commercial cultivar from Veneto allows to discover and highlight some features useful to produce food supplements recommended in the treatment of obesity and diabetes.

This study showed that only two of the tested cultivars (Fasolu Pintau and Faitta a Cavanedda) are not suitable as starting material to produce food supplements due to the absence of inhibitory activity against porcine, and above all, against human amylases, while all cultivars have α-glucosidase inhibitory activity.

The cultivar Nieddone, results to be the most interesting variety, due the presence in its extract of inhibitory activity against both α-amylase and α-glucosidase enzyme, but most importantly, for the lack of haemagglutinin activity, an antinutritional factor that prevents the consumption of raw beans.

Molecular studies conducted on the reverse transcribed RNA in cDNA showed the expression of α-AIs in all the cultivars analysed. Having deduced part of the primary structure of the proteins, we speculate that the proteins PV152-α-AI and PV20-α-AI, share more molecular characteristics with isoform α-AI-4 rather than with isoform α-AI-

1. A future goal will be to complete cDNA sequencing to increase understanding of the primary structure of these proteins. In fact, the mechanistic characterization suggests that the α-AI had two main hotspots in the interaction with α-amylase enzyme but only for the cultivar Granino (ACC.28) it has been reconstructed an aminoacidic fragment that includes one of these sites. The PV28-α-AI does not differ from the aminoacidic reference sequence used for the analyses (PO28731) in the Y186 position.

However, aminoacidic differences could be present at the level of the first hotspot site (D38) not yet identified in this work. These differences could explain for example why the cultivar Fasolu Pintau and Cavanedda have not inhibitory activity although the α-AI is expressed in both of them.

The achievement of this objective could be important both for increasing the biomolecular knowledge of these proteins and for the development of further studies in the biotechnological field aimed at the expression of more promising peptides or proteins from a biochemical point of view.

3. Purification and characterization of proteinaceous thermostable α-amylase inhibitor from Sardinian common bean Nieddone cultivar (*Phaseolus vulgaris* L.)

The increasing need to find new solution for the treatment of obesity and diabetes led to the development of new drugs and food supplements that could reduce the carbohydrates absorption. Many starch blockers, based on common bean proteinaceous inhibitors against digestive enzymes such as α-amylase, are already present in the commerce [2]. The importance in the clinical setting and the possible economic implication were decisive in the choice to extract and purify α-amylase inhibitor from the most promising common bean cultivar identified during the first phase of study.

Nieddone cultivar appeared to be the most promising cultivar for its inhibitory activity against digestive enzymes but above all for the lack of hemagglutination power (see above).

Different techniques were then used for the extraction and the purification of the inhibitor. Moreover, a biochemical characterization of the inhibitor has been performed. This aspect is important to better understand the inhibitor properties and above all the usefulness in the production process of new food supplements enriched with the purified inhibitor.

3.1 Materials and methods

3.1.1 Chemicals

2-Chloro-4-nitrophenyl-α-D-maltotrioside (93834-100MG), *p*-nitrophenyl-α-D-glucopyranoside (487506-1GM), α-amylase from porcine pancreas (Type VI-B A3176-2.5MU), PNGase F from *Elizabethkingia miricola* (P7367) and α-Glucosidase from *Saccharomyces cerevisiae* (Type I G5003-1KU) were purchased from Sigma–Aldrich (Milan, Italy). All other reagents used were of the highest grade available, purchased from Sigma–Aldrich (Milan, Italy) and used without further purification.

3.1.2 Plant materials

As described in Chapter 2 the Sardinian cultivar used to purify and characterize the inhibitor is called Nieddone, coming from Ploaghe, a little Sardinian village. It is part of a wider germplasm collection by the Sardinian Agricultural Agency (AGRIS, Cagliari, Italy) collected with the accession number ACC.177.

As reported in Chapter 2 the α-AI gene sequence has been deposited in NCBI GenBank with the code OP530245.

3.1.3 Purification of amylase inhibitor

Protein extraction

Legume seeds were homogenized to a fine powdery flour in a blender. The flour was then immediately extracted and used for the extraction. Briefly, aliquots of 2 grams were suspended in 10 mL Bis-Tris buffer pH 6.5 mM, NaCl 0.1 M buffer; then stirred for 1 hour and centrifuged at 12000 rpm for 10 minutes at 4°C. The extraction has been also performed in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor.

Low-resolution purification techniques

The supernatant after extraction was collected and heated to denature heat-labile proteins for 10 minutes at difference temperatures and then centrifuged at 12000 rpm for 30 minutes at 4°C.

Alternatively, it was brought to 45–80% saturation with ammonium sulphate and centrifuged at 12000 rpm for 30 minutes at 4°C.

Solvent precipitation was also used adding, dropwise, the raw extract to stirring iced solvent (ethanol or acetone) in a 1:50 volume ratio. The solution was then centrifuged at 12000 rpm for 20 minutes at 4°C and the supernatant discarded. After 24 hours (during

which the residual solvent evaporated) the pellet was resuspended in 10 mL of starting buffer.

Ion Exchange Chromatography

The sample was applied to fast protein liquid chromatography (FPLC) in Akta Start (Cytiva, Milan, Italy) using anion exchange chromatography with HiTrap Q HP 5mL column Start (Cytiva).

Column was equilibrated with 5 column volumes (CVs) of Bis-Tris pH 6.5 10 mM, NaCl 0.1 M buffer at 4 mL/min flow rate. Sample (8 mL) was injected, and unbound proteins were washed with 4 CVs buffer. Elution was then performed with a 20% step gradient in B buffer (Bis-Tris pH 6.5 10 mM, NaCl 1M), and 1 mL fractions being collected automatically. After 4 CVs, a linear gradient 100% B in 2 CVs was then used to wash the column.

Size Exclusion Chromatography

HiLoad 16/60 Superdex 200 pg (Cytiva) column was used (120 mL CV). This was equilibrated with 4 CVs Bis-Tris pH 6.5 10 mM, NaCl 0.1 M, CaCl2 5 mM buffer at 1 mL/min flow rate. Sample (4 mL) was injected, and 1 mL fractions were collected automatically in isocratic elution.

MW of the purified protein was determined by comparison with MW standards (Gel Filtration Standards, cat. n. 1511901, Bio-Rad, Milan, Italy) under the same operational SEC conditions.

Purity of the sample was verified by SDS–PAGE using 12% polyacrylamide gels using Laemnli protocol [219], and by total protein determination using Coomassie Brilliant Blue G-250 method [175].

3.1.4 α-Amylase and α-glucosidase inhibition assays

As described in Chapter 2, the inhibition of α-amylase activity was determined using 2-chloro-p-nitrophenyl-α-D-maltotrioside (CNP-G3) as substrate. α-Glucosidase inhibition assay used the same experimental conditions of CNP-G3 protocol. The substrate used was p-nitrophenyl-α-D-glucopyranoside (pNPG) 2.25 mM.

3.1.5 Hemagglutination assay

Human blood 2 mL was centrifuged at 2000 g for 3.5 minutes, supernatant was discarded, and the cell pellet was washed three times with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Red blood cells (RBC) pellet was suspended to 50% (v/v) with PBS buffer, stabilized with 0.01% natrium azide and stored at 4°C. The life span of RBC was up to three weeks.

One aliquot of RBC 10% was prepared, and each raw extract was used at different serial dilutions (pure, ½, ¼, 1/8, 1/16….as far as 1/512.)

For each combination of RBC and protein concentration, the RBC sample was pipetted onto a glass microscopy slide and mixed in a 10 µL:10 µL ratio with the protein solution. The mixture was incubated for 5 minutes and observed using an inverted fluorescence microscope BX81 (Olympus, Segrate, Italy). Images were captured with a charge-coupled device camera (Cohu, San Diego, CA, USA). In the control experiment, 10 µL of the PBS buffer was incubated with RBC.

Results are expressed as the Minimum protein Concentration able to Agglutinate sample (MAC, mg/mL).

3.1.6 α-Amylase from *Tenebrio molitor*

Insect α-amylase was obtained from *Tenebrio molitor* larvae. This insect feeds on stored grains and food in general, being usually considered a pest. Aliquots of 1 g larvae were grinded with 6 mL Bis-Tris buffer pH 6.5 mM, NaCl 0.1 M, as already described [220]. This homogenate was then centrifuged at 8,000 g for 30'. The supernatant was used as crude insect α-amylase preparation for activity assays as described above.

3.1.7 Inhibitor characterization

The activity at different pH values was determined repeating the catalytic assay in the presence of different 0.5 M McIlvaine buffers ranging from pH 3 to 9 at 37°C. Thermo-stability was assayed by incubating the enzyme at the indicated temperature for the indicated time. Then the samples were rapidly brought to 4°C and the remaining catalytic activity was assayed as previously described.

Michaelis–Menten kinetic parameters were calculated using CNP-G3 ranging 0.1-5 mM and using GraFit 7 (Erithacus Software, London, United Kingdom).

3.1.8 LC-MS

Deglycosylation was performed according to manufacture instructions. Aliquot of 50 µg protein was denatured with 0.2% SDS and 100 mM 2-mercaptoethanol at 100°C for 10'. Then 5 EU of PNGase F was added. After 3h incubation.at 37°C, the reaction was stopped by heating at 100°C for 5'.

3.1.9 Statistical analysis

GraFit 7 (Erithacus Software, London, United Kingdom), R 2.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and GraphPad INSTAT (GraphPad Software, San Diego, CA, USA) were used for data analysis. One-way analysis of variance (ANOVA)

and the Bonferroni multiple comparison test were used to assess the statistical significance of the differences. All the analyses were performed at least in triplicate (unless otherwise stated), and the data are reported as mean ± standard error of the mean (SEM).

3.2 Results and discussion

In the previous Chapter a screening of different common bean cultivars has been carried out to identify the most promising variety to produce food commercial supplement. Our analyses showed that Nieddone is the most interesting variety for its quite high inhibitory power against digestive enzymes and for the lack of hemagglutination activity. Considering such evidence, in the present Chapter extraction, purification, and characterization of α-AI isolated from Nieddone cultivar is described.

3.2.1 Low resolution techniques for α-AI extraction.

In a first step low resolution techniques were used for the inhibitor extraction in order to keep production costs as low as possible, obtaining a final inexpensive food supplement [221] . This is a crucial requirement for a potential nutraceutical marketable product. Firstly, the common bean seeds were ground and suspended in Bis-Tris buffer pH 6.5 10 mM, NaCl 0.1 M. Different extraction conditions were tested. The influence of protease inhibitor, extraction time and temperature on the α-AI activity have been evaluated as the crucial parameters of the process.

As described in Chapter 1, α-AI is translated as a pre-protein, proteolytically activated at Asn77. So, the addition of a well-known protease inhibitor (phenylmethylsulfonyl fluoride, PMSF) was tested at 4°C and 25°C to assess the presence of an eventual proteolytic activation during the first phases of purification (Figure 3.1).

Only slight proteolytic activation occurs during the extraction, but no statistical differences were found even after 24 h between the inhibitory activity in the sample extracted with or without PMSF, suggesting that no proteolytic activation occurs in the first phases of purification.
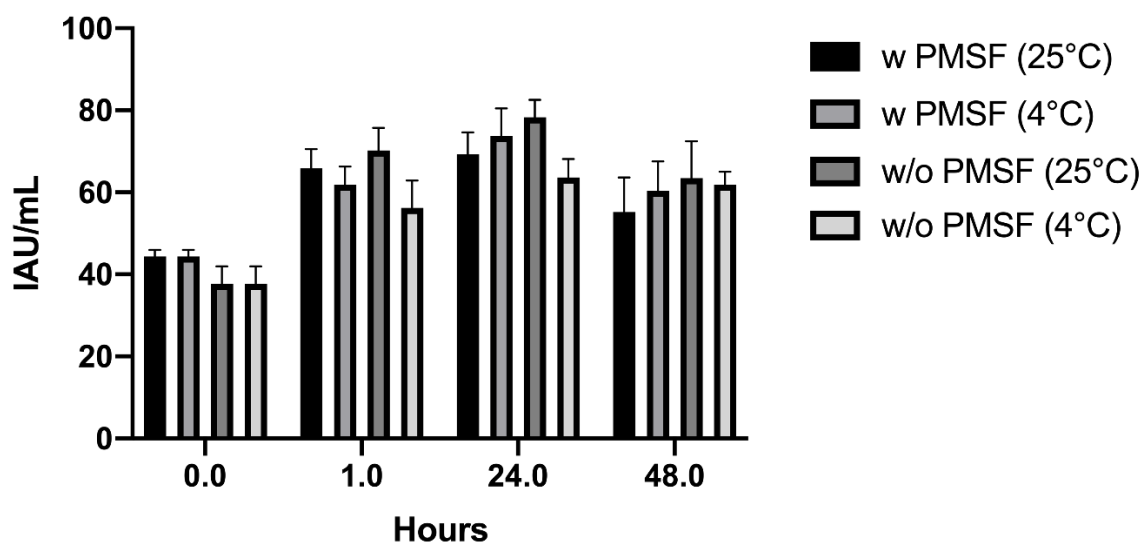
Figure 3.1 α-AI activity recorded after different extraction conditions.

α-AI activity appears in any case to be higher after 24 hours than after 1 hour, but no significant increase was recorded for longer times of incubation (24 hours or 48 hours). Thus, the stirring was performed for 1 hour in all experiments.

Similarly, no significant differences were observed between 4°C and 25°C. So, to avoid microbial contamination all the extraction procedures were performed at 0°C-4°C in accordance with what previously reported in literature [29,196].

The suspension obtained was then centrifugated, as reported above, and the supernatant collected.

In accordance with Bharadwaj, Gupta and Le Berre-Anton [29,50,196] the sample was then subjected to several precipitation techniques, including 45-80% saturation with ammonium sulfate and cold organic solvents (ethanol and acetone) to improve purification fold.
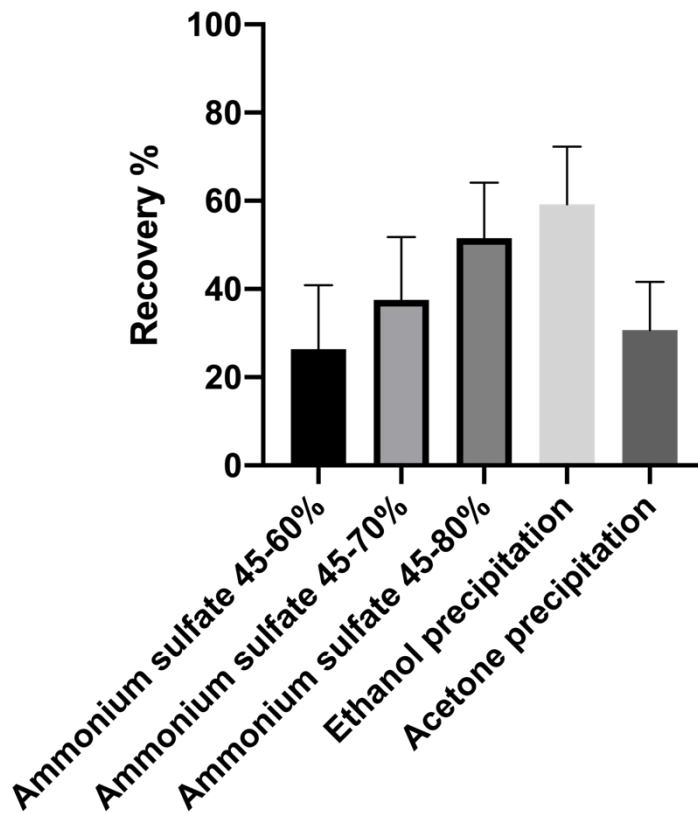
Figure 3.2 Comparison of recovery % α-AI extraction after ammonium sulfate saturation and organic solvent precipitation.

Different ammonium percentage were tested, but as shown in Figure 3.2 the better recovery extraction (the retained enzymatic activity in the purified sample) was obtained with the ethanol precipitation.

Considering that α-AI is a thermostable protein, the supernatant was also heated for 10 minutes at 60 and 70°C, as previously suggested [50,63], to reduce the total proteins maintaining a high percentage of α-AI.

Two different set of experiments were done. The heat treatment was performed at 60°C and 70°C and the resulting inhibitory activity compared with the activity recorded after ethanol precipitation. As shown for instance in Figure 3.3, the thermal denaturation was tested at 60°C both after and before ethanol treatment.
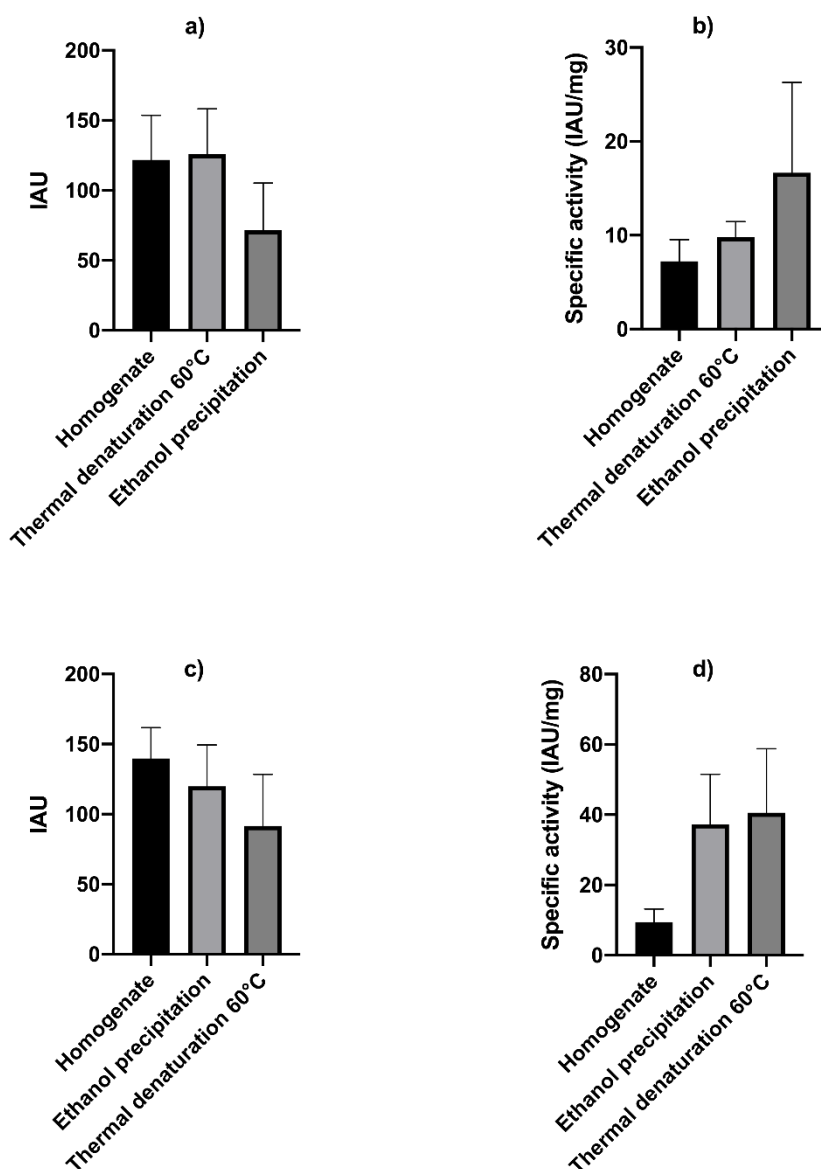
Fiure 3.3: Graphical rapresentation of α-AI activity (IAU) and specific activity(IAU/mg): (a;b) the termal denaturation precedes the ethanol precipitation; (c;d) the ethanol precipitation precedes the thermal denaturation.

In Figure 3.3a the thermal denaturation precedes the ethanol precipitation. The thermal treatment allowed to recover almost all the inhibitory activity, whereas the ethanol treatment led to a loss of about 50%. However, the thermal treatment did not allow to increase the specific activity (Figure 3.3b).

When the ethanol precipitation preceded the thermal denaturation, a similar behaviour was observed, since only the organic solvent significantly increased the specific activity (Figure 3.3d).

The same experiment was carried out at 70°C. Also, in this case the heat treatment did not improve the specificity activity (data not reported). For these reasons, it has been established to use only ethanol precipitation as low-cost purification technique. Such choice allowed to increase purity of sample quickly, without requiring any complex instrumentation of operator expertise. Besides, the procedure was also quite inexpensive since the ethanol used could be efficiently distilled and recovered for more purification cycles.

The precipitation was re-dissolved in the starting buffer and centrifugated before proceeding further the purification.

3.2.2 High resolution techniques: purification of α-AI

The α-AI from Nieddone has been purified by two purification steps performed with fast protein liquid chromatography (FPLC) using an Akta Start system (Cytiva, Milan, Italy). This instrument is preparative chromatography system, allowing to automatize protein purification, reducing time process and leading to a more precise and reliable analyses.

In the first step the ethanolic extract was loaded in an anion exchange chromatography Q-Sepharose column previously equilibrated with Bis-Tris pH 6.5 10 mM, NaCl 0.1 M, and then washed with buffer B (Bis-Tris pH 6.5 10 mM, NaCl 1 M). Bound proteins were eluted firstly with a 20% step gradient in B buffer, and then with a 20-100% linear gradient with the same B buffer.

The elution pattern showed only one peak with α-AI activity, eluting with a low ionic strength (20% B), as shown in Figure 3.4. $CaCl_2$ 5 mM was then added in the active fractions to improve inhibitor stability [79,80].

Finally, the active fractions were collected and subjected to a Size Exclusion Cromatography (SEC) using a cross linked agarose resin column. Binding proteins were eluted with an isocratic gradient using bis-Tris pH 6.5 10 mM, NaCl 0.1 M, $CaCl_2$ 5 mM buffer. A main peak showing biological activity was eluted around 80 mL V_e (Figure 3.5).

Traces of inhibitory activity were also detected in other eluted peaks, but this was quite unstable. Such evidence could be possibly due to some form of protein degradation or aggregations.
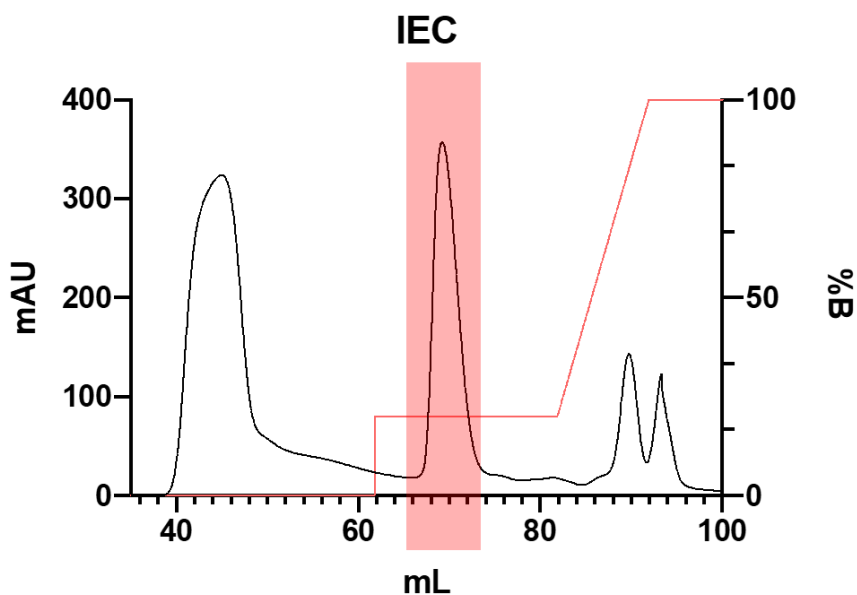
Figure 3.4 Elution profile of α-AI from P. vulgaris *Nieddone cv on IEC Q-Sepharose column chromatography.*
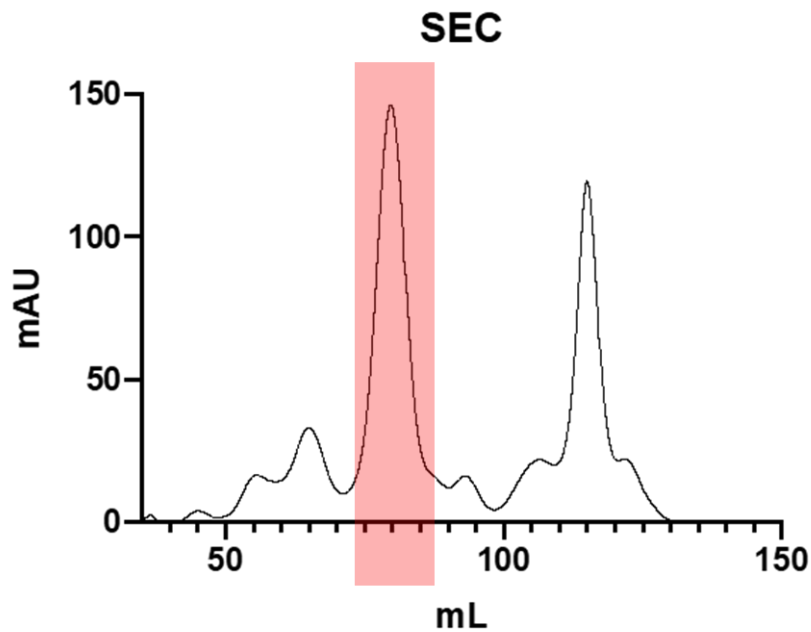
Figure 3.5 Purification profile of α-AI from P. vulgaris *Nieddone cv on a SEC cross linked agarose resin column.*

All the results of the extraction and purification procedure are summarized in Table 3.1.

Medie	Amylase inhibiting activity (IAU)	Total protein (mg)	Reco-very	Specific activity (IAU/mg)	Purifica-tion fold	Aggluti-nation
Raw extract	242	60.22	100%	4	1.0	Neg.
Solvent precipi-tation	147	15.68	61%	9	2.3	Neg.
IEC	59	3.24	24%	18	4.5	Neg.
SEC	80	0.24	33%	328	81.8	Neg.

Table 3.1 Purification table of P. vulgaris Nieddone cv α-AI

Each purification steps led to a considerable increase in specific activity and purification fold (Figure 3.6). After the final purification step, the enzyme was purified over 80-fold

with a recovery of 33%. The specific activity of the purified α-AI (328 IAU/mg) was significantly higher than that of α-amylase from raw extract (4 IAU/mg). The absence of agglutination power was confirmed after each purification passages.
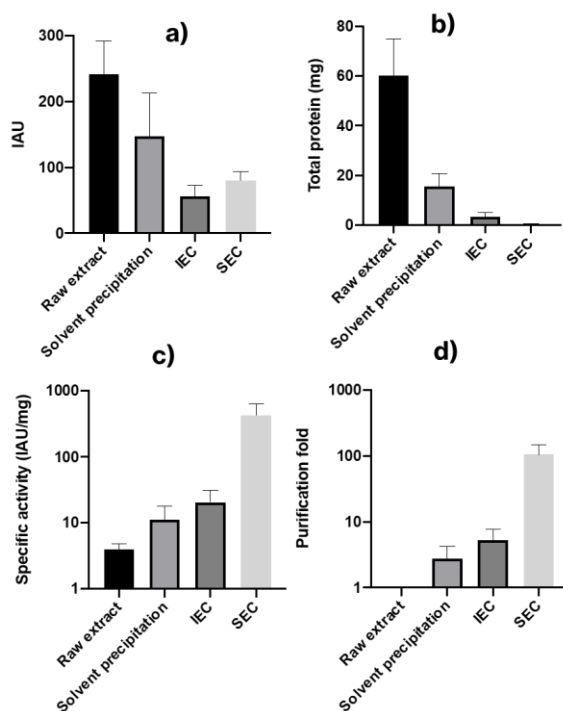
Figure 3.6 Graphic rapresentation of purification steps of α-AI from P. vulgaris *Nieddone cv.*

3.2.3 α-AI characterization

All the purification steps were analysed by denaturing PAGE to evaluate the increase in purification degree. As shown in Figure 3.7, the purified inhibitor gave two bands close to 15 kDa molecular standard, confirming the existence of two subunits (theoretical MWs for α and β chains are respectively 8 and 15 kDa [58]).
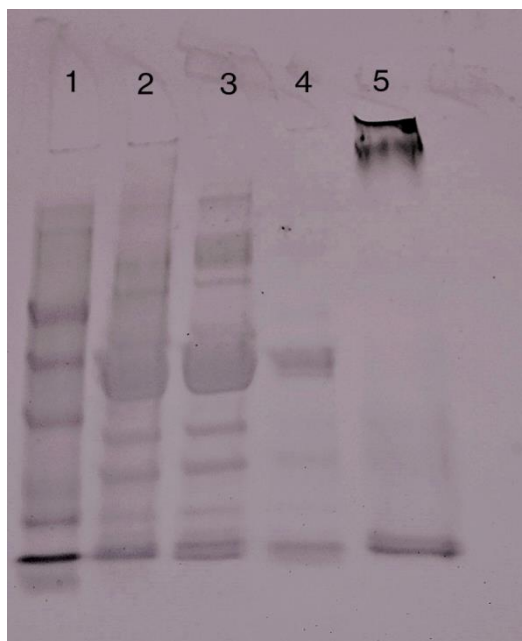
Figure 3.7 SDS-PAGE of α-AI from P. vulgaris *Nieddone cv.during purification steps. Molecular weight standard is reported in line 1; α-AI raw extract is reported in Line 2; after the ethanolic precipitation in Line 3, after Q-sepharose ion exchange cromatography in Line 4, after the size exclusion cromatography in Line 5. SDS-PAGE was performed following Laemnli protocol*

Besides the bands belonging to α and β subunits, SDS-PAGE showed also that some proteins were not able to enter into the gel, resulting in an unresolved and undefined band in the proximity of the well (Figure 3.6). Such evidence could confirm the tendency of α-AI to form aggregates, as already described in literature [34], and as also suggested by SEC (see below).

The previously described tetrameric association of these 2 subunits [50] was confirmed by size exclusion chromatography. The elution volume of the purified protein (Figure 3.5) in fact has been compared with a mixture of molecular weight standards (Figure 3.8a) estimating a molecular weight of 42 kDa (Figure 3.8b), in accordance with the tetrameric $\alpha_2\beta_2$ structure [222].
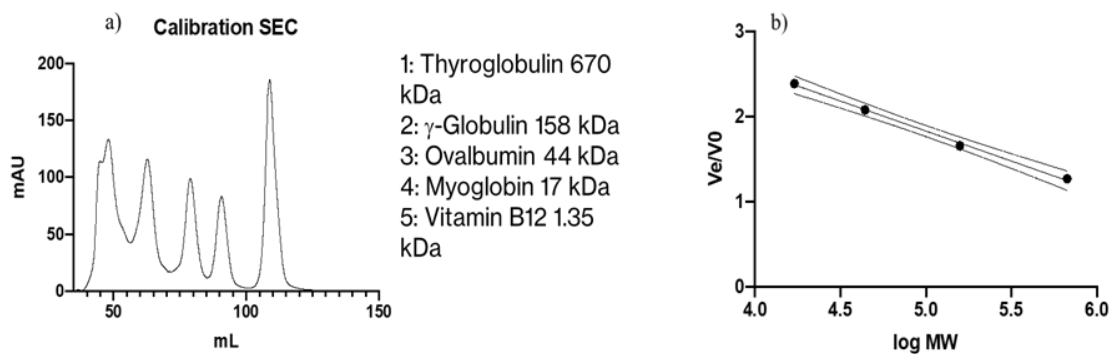
Figure 3.8. Calibration of SEC with MW standards to determine α-AI MW.

In literature a great variability in *P. vulgaris* α-AI molecular weight was recorded.

Only one subunit of 36 kDa was identified in *P. vulgaris* in 2008 [223]. Quite in accordance with monomeric structure described in *Mucuna pruriens* (25.6 kDa) [29] and *Secale cereale* (13.7 kDa) [224].

Three subunits were described by different authors in *P .vulgaris* (α subunit ranged from 7.8 kDa to 15.5 kDa, β ranged from 14 kDa to 18.6 kDa and γ ranged from 22 kDa to 26.3 kDa) [196,225], and four subunits ranging from 14 to 20 kDa were described in a study on *P. vulgaris* inhibitor in 2011 [226].

Instead, two α-amylase inhibitors were identified in the tepary bean (*Phaseolus acutifolius* A. Gray) [30]. One of these (αAI-Pa2) was very similar to α-AI being composed of α and β subunits but neither had inhibitory activity against α-amylases of mammalian, bacterial or fungal origin, but only against insect α-amylase.

Moreover, two prominent amylase inhibitor activity bands (AI1 and AI2) were detected in the seeds of *Achyranthes aspera* identified [220]. The molecular weight of the purified inhibitor was about 6 kDa ad it is active against insect α-amylase.

However, Literature agrees on the tetrameric α$_2$β$_2$ structure for α-AI from *P. vulgaris* with a total 44/45 kDa MW [53,55,213].

This variability could be linked to the different technique used for the determination of molecular weight or to the different cultivar and species analysed.

The purified α-AI was the tested to evaluate the trend of its activity during prolonged storage. It has been tested for 36 days. As shown in Figure 3.9 the inhibitory activity looks quite stable over time. After 36 days only about 25/30% of its initial activity was lost. This stability is a desirable feature because it would allow to exploit the inhibitory power during the producing process of possible food supplements, even without expensive and time-consuming procedure of solvent removal (i.e. freeze-drying).

For what in our knowledge no similar storage stability has been studied up to now for α-AI. The long-lasting α-amylase and α-glucosidase inhibitory activity was studied for instance in white tea stored for 1, 3 and 5 years.[227]. However, in this case the inhibitory activity decreased with the decreasing of total polyphenolic content pointing out that these are probably not proteinaceous inhibitors.

Further investigations are needed to understand if this stability is confirmed also for α-AI extracted from other cultivars.
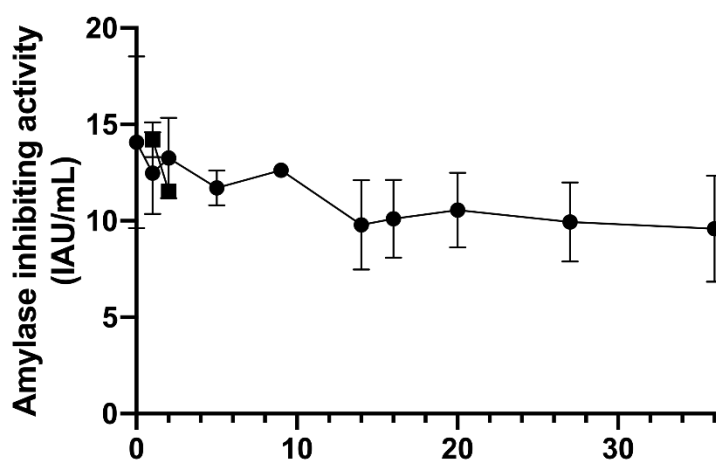
Figure 3.9: Trend over time of α-AI from Nieddone cv activity. The time course was observed at regular intervals for 36 days.

3.2.4 Activity of α-AI from Nieddone cv against mammalian, bacterial, and insect α-Amylases

Purified α-AI was then tested to study several operational characteristics, such inhibitory activity against α-amylase enzyme from different organisms and effect of pH and temperature conditions.

The purified inhibitor activity was tested against four α-amylase enzymes from different sources (porcine pancreas, human saliva, *Bacillus subtilis* and *Tenebrio molitor*).
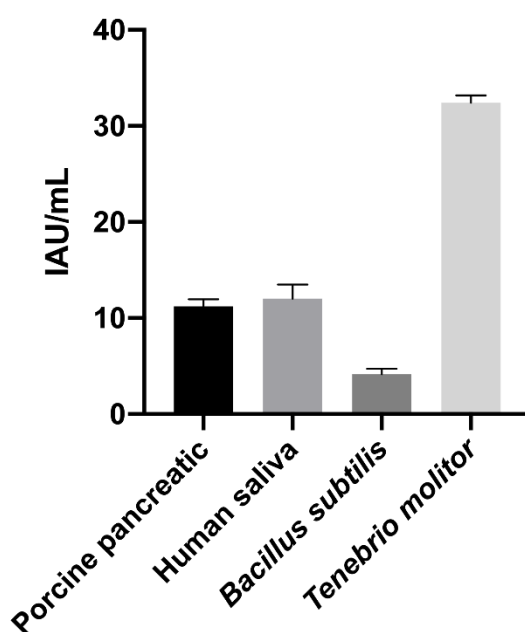
Figure 3.10: Inhibitory power of α-AI purified from Nieddone cv against pig, human, bacterial and insect α-amylase enzyme.

As shown in Figure 3.10 α-AI from Nieddone cv has the highest affinity against the insect α-amylase *Tenebrio molitor,* about 3-fold more susceptible than human salivary amylase and porcine pancreatic α-amylase. The purified sample showed inhibiting activity also against α-glucosidase enzyme (3.8 ± 0.8 IGU/mL).

This result is in contrast with what observed for the *Moringa oleifera* α-AI extract, which had more inhibitory activity against human α-amylase than *Callosobruchus maculatus* insect larvae [228].

In any case the presence of inhibitory activity against human and insect α-amylase confirmed that the purified inhibitor corresponds to α-AI1 isoform [40].

The highest affinity of α-AI from *P. vulgaris* for insect α-amylase had already been observed also for other species (*Tribolium castaneum, T. confusum* and *Sitophilus oryzae*) [229].

Recently, many authors have confirmed the role of α-amylase inhibitor from different plant species against α-amylase enzyme of insects [230,231], pointing out that the administration of α-amylase inhibitors could also represent a valuable approach for biological pest control.

Therefore, α-AI from Nieddone cultivar could also have a crucial role for fighting harmful pests that infect legume of significant socioeconomic and nutritional importance.

3.2.5 Time and temperature effects on α-AI

As above mentioned, the inhibitory activity was recorded for 12 mins with the CNP-G3 assay that involved 10 mins of α-amylase enzyme/α-AI preincubation before the substrate addition.

In Literature different preincubation values for common bean α-AI have been reported, since the maximum inhibition was achieved after 10 mins preincubation time [50], or after 40 minutes [63] and 120 minutes [77]. These differences could be dependent on the cultivar analysed or on the different assay used for recording α-amylase activity. In fact, unlike the method employed here, in the previous studies the DNS assay was used.

We observed that the inhibitory activity of our purified inhibitor increases if pre-incubation is prolonged over time (Figure 3.11). Reaching a complete inhibition with 120 mins preincubation, in accordance with Power and Whitaker [77].
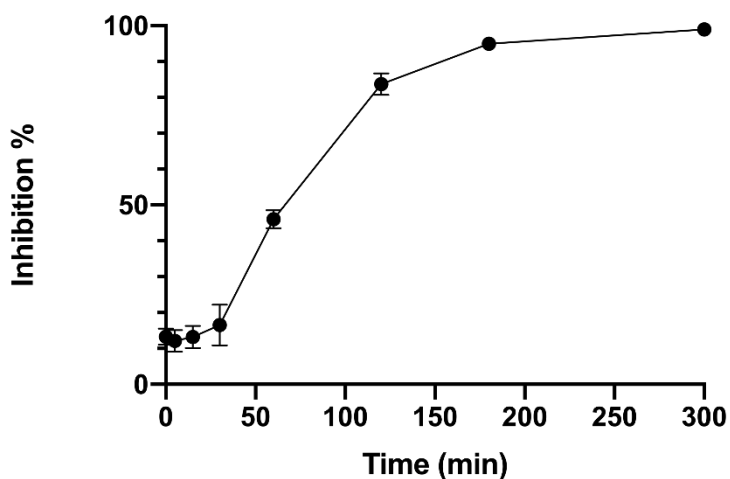

Figure 3.11: Trend of inihibition of α amylase enzyme by α-AI with different preincubation times.

The preincubation time is important if the possible use of the inhibitor for commercial purposes is considered. These data suggest that a food supplement based on this inhibitor should be taken two hours before the meal to achieve the maximum inhibitory activity.

The effect of temperature on the α-AI optimum was studied by incubating the purified inhibitor at different temperature ranging from 26°C to 45°C (Figure 3.12). It is well known that an increase in temperature exerts a double effect of proteins, speeding up the reaction in accordance with Arrhenius equation, but at the same time inactivating proteins destabilizing native state.

As shown in the graph the α-AI activity showed an optimum temperature obtained at 40 °C.
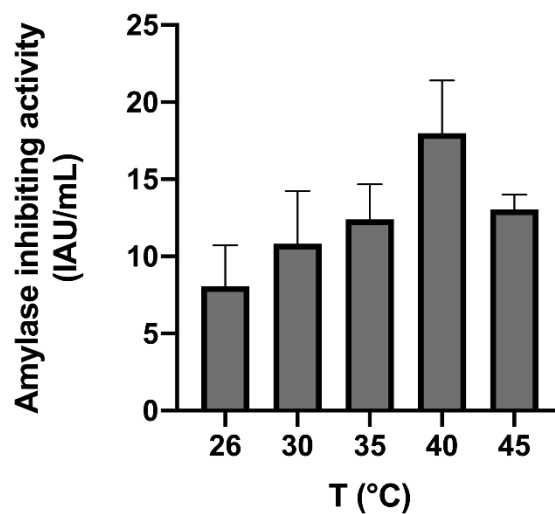
Figure3.12: Effect of temperature on the purified Nieddone inhibitor: the optimum temperature was observed at 40°C.

Similar optimum values were also found for α-amylase inhibitor extracted from *Mucuna pruriens* [29] and in kidney bean where the optimum was reached at 37 °C [50,63]. This is a desirable condition for the use of inhibitor to produce dietary supplements because it reaches optimum at physiological body temperature.

For what concerns the α-AI heat stability, interesting data emerged from this study. It is known from Literature that plant proteinaceous inhibitors showed quite remarkable temperature stability.

For example, the α-AI purified from *P. vulgaris* (Purola cultivar) was stable at 90°C preserving about 90% of its activity [206].

The α-amylase inhibitor purified from *Mucuna pruriens* (*Fabaceae* family) was found to be heat-stable, retaining about 80% of its activity after 30 minutes at 65°C [29].

The thermal stability of α-amylase inhibitor was also observed in different varieties of *Triticum durum* [232]. In this case, both varieties were stable below 80°C with a

maximum inhibitory activity ranging from 40 to 50 degrees. When brought to the boil, the purified α-AI lost about 75%-55% of its activity.

Another purified proteinaceous α-amylase inhibitor extract from *Moringa oleifera* shows heat temperature stability, preserving about 50% of activity for one hour at 70°C [228]. Our data seems to confirm this trend. In fact, we tested our purified sample to different temperatures ranging from 50°C to 100 °C and the inhibitory activity was recorded at different time intervals as shown in Figure 3.13b.
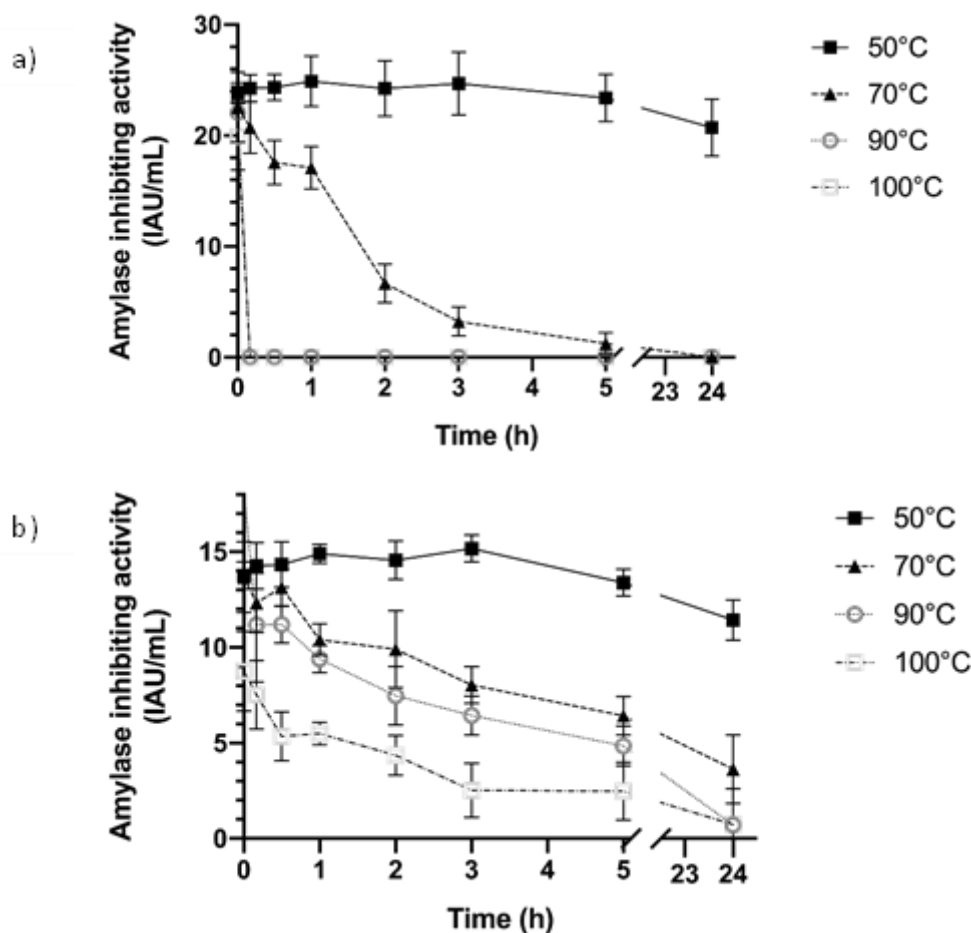
Figure 3.13: Effect of temperature on inhibitor stability. α-AI activity was assay to different temperatures and the residual activity recorded at varius time intervals. a)the stbility of inhibitor from raw extract; b) the stability of puridied extract from Nieddone cv.

We also compared purified α-AI temperature stability with the Nieddone bean raw extract (Figure 3.13a).

The two samples showed very different behaviour. Both were stable at 50°C in a time interval between up to 24 hours (more than 90% activity retained). But the inhibitory activity rapidly decreased at 70°C after one hour in the raw extract (about 75% activity retained). Its inhibitory activity was completely destroyed after 10 minutes at 90 and 100°C in accordance with what previously observed [50,63,82].

On the other hand, the purified α-AI was incredibly more stable under the same conditions (Figure 3.13b), maintaining for instance about 25% of its activity, even at 100 °C for 5 hours. The inhibitory activity not completely disappeared even after 24 hours, showing even better performance than other proteinaceous plant inhibitors [29,228].

It is possible for a purified protein to be more stable at a given temperature than a crude extract of the same protein. Crude extracts are made up of a mixture of different proteins and other biomolecules, which can lead to instability due to interactions between these molecules. In contrast, a purified protein is isolated from these other molecules, which can make it more stable. This desirable property has been also described in the Purola cultivar (*P. vulgaris*). Also in this case the activity of the purified inhibitor was significantly more stable than the raw extract [206].

Our data shows that also Nieddone purified α-AI is much more thermostable than the same protein present in the raw extract. Such evince could suggest that the costs of purification could be justified by this increased stability. In fact, the resistance to high temperatures is crucial parameter for producing inhibitor-enriched foods that need to be cooked before being consumed such as crackers, biscuits, or pasta. This specificity would

allow to produce palatable dietary food. However more investigations are needed to identify a procedure to further stabilize the inhibitory activity at the higher temperatures.

3.2.6 Effect of pH

As shown in Figure 3.14, porcine pancreatic α-amylase enzyme showed a pH optimum at pH 5. When the catalysis was instead performed in the presence of α-AI, by incubation using different 0.5 M McIlvaine buffers, two optima for inhibition were observed at 5 and 6.5 (Figure 3.14).

Two optima (5 and 6.9) were previously found for local Himalayan bean [196] while only one pH optimum (ranging from 4.5 to 6.9) was found in Japan and kidney beans [63,80,222,233].

These differences could be related to the different experimental conditions or to the different α-amylase activity assay used (all the mentioned authors used DNS assay).

In fact a shift in the pH optimum was observed in presence of different substrates [234]. Porcine pancreatic α-amylase hydrolyse starch with a pH optimum of 6.9, but the optimum shifts to 5.9 when low molecular weight substrate as CNP-G3 is used, as in this study. However, we chose to perform the CNP-G3 assay at pH 6.5 in accordance to Literature [198]. This choice is related to the *in vivo* conditions of α-amylase enzyme. In fact, *in vivo* it works with starch as the substrate under conditions closer to neutrality than to acidic environment.
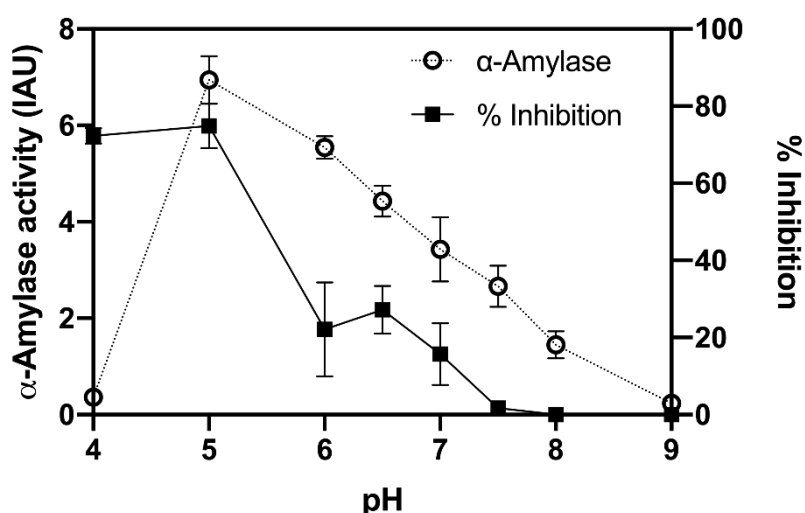
Figure 3.14: Effect of pH on the activity of α-amylase enzyme and on the activity of the purified inhibitor form Nieddone cv.

3.2.7 Kinetic analysis

The kinetic analysis of the enzyme in the presence of the CNP-G3 substrate showed a behaviour typical of a kinetics according to the Henri-Michaelis-Menten (H-M & M) equation. The K_M value was 0.85 mM and the V_{max} was 30.9 µM / min (Table 3.2). When the activity of the enzyme was carried out in the presence of inhibitor concentrations ranging between 0.25 and 0.50 µM an increase in the value of K_M and a decrease in V_{max} were observed. In particular, at the concentration of 0.50 µM α-AI a value of the K_M^{app} = 1.10 and a V_{max}^{app} = 21.8 were measured. These values agreed with a mixed-type inhibition measured according to the plot of reciprocal doubles in the absence of the inhibitor and in the presence of partially inhibited catalysis (Figure 3.15). Previously, a mixed non-competitive inhibition was described for α-AI from kidney bean, using porcine pancreatic amylase [222]. It should be noted that in these experiments, starch was used as the substrate.
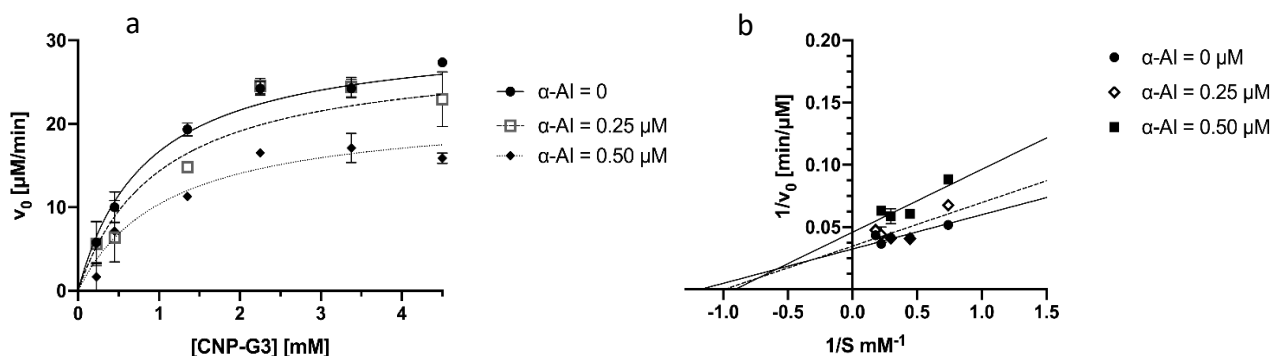
Figure 3.15:a)Kinetics of inhibition of α-amylase by the purified inhibitor from Nieddone cv without α-AI (●),with 0.25 μM α-AI (□) with 0.50 μM α-AI (◆) ; b) Lineaweaver-Burk plots of α-amylase enzyme alone (●), inhibited with with 0.25 μM α-AI (◇) and with 0.50 μM(■).

	α-AI = 0	α-AI = 0.25 μM	α-AI = 0.50 μM
V_{max} (μM/min)	30.9	28.8	21.8
K_M (mM)	0.85	1.01	1.10

Table 3.2: Determination of kinetics parameters for α-AI purified from Nieddone cv.CNP-G3 was used as substrate.

Our results are not in full agreement with those previously reported by other authors who described a non-competitive inhibition for α-AI of *P. vulgaris* var. Great Northern using starch as the substrate [63]. A non-competitive mixed inhibition of a digestive amylase in the presence of a proteinaceous inhibitor from *Moringa oleifera* was also recently confirmed [228].

Compared to the data reported in the literature, there is no general agreement on the type of amylase inhibition shown by protein inhibitors of plant origin. This should not seem strange considering the complex mechanism that leads to the release of different peptides with amylase inhibitory activity. Furthermore, it is likely that the different type of reversible inhibition we found may be due to the different substrate we used in our enzymatic assay. In fact, a mixed non-competitive inhibition suggests that the substrate interact with

the enzyme in more than one site. So, it would be not surprising if different substrates could lead to different kinetic results.

3.3 Conclusions

A combination of low-resolution techniques and chromatographic steps was used to purify the α-amylase inhibitor from the common bean seeds (*Phaseolus vulgaris*) of the Sardinian cultivar Nieddone. This procedure could be also apt for scaling-up of commercial preparations, since it is quite inexpensive, there is no need for high operator expertise and it could be in great part automated.

The purified inhibitor has a tetrameric structure with a molecular weight of about 42 kDa, confirm the $\alpha_2\beta_2$ association already reported in Literature.

The α-AI maintained its activity over time, remaining stable for at least 30 days at 4°C.

α-AI was effective against pig, human and insect α-amylase enzyme. This aspect could play an important role not only in the management of nutritional and diabetes-related disorders, but it could also open new paths in the fight against pests.

A mixed-type inhibition of α-amylase was observed, thus suggesting that the inhibitor can bind to the enzyme at more than one site and inhibits its activity through more than one mechanism.

The temperature optimum of the inhibitor is about 40°C and it has two pH optima (5 and 6.5).

It is very interesting its resistance to thermal denaturation (about 25% of activity retained, even at 100 °C for 5 hours). The purification allowed to significantly enhance this feature, since the raw extract lost completely its activity after 10 minutes at 90 and 100 °C, highlighting the importance and the usefulness of the purification procedure.

This high temperature tolerance, the duration of inhibitory activity over time, the broad pH stability and the fact that no agglutination activity is present in the purified protein, indicate the purified α-AI effectiveness in the production of food supplement resistant to different gut conditions or the possibility to produce cooked foodstuffs enriched with α-AI.

On the whole, these data pave the way not only to the production of new food supplements enriched with purified α-AI useful in the management of obesity and diabetes treatment, but they also suggest that the purified α-AI could be a good potential candidate for insect pest management.

4. Final considerations

Phaseolus vulgaris α-amylase inhibitor (α-AI) is a protein that recently gained commercial interest. This protein inhibits mammalian α-amylase activity, reducing carbohydrates absorption. Numerous studies reported the efficacy of preparations based on this protein on the control of glycaemic peaks in type-2 diabetes patients and in overweight subjects. A positive influence in microbiota regulation has been also described. Besides, it can be also employed to control pest infestation since it is also able to inhibit insect amylase. In this context, the development of novel inhibitors from commercial legumes is an important task to preserve local biodiversity and rediscover partially forgotten species and cultivars.

In accordance, a screening was performed on 9 rare Sardinian common bean varieties and on one commercial cultivar from Veneto, identifying some features useful for producing dietary supplements effective in obesity and diabetes management.

Even though two cultivars have not α-AI inhibitory activity (Fasolu Pintau and Faitta a Cavanedda), molecular studies showed that the α-AI gene is expressed in all cultivars.

Besides, the partial nucleotide sequence of α-AI gene was identified with the degenerate hybrid oligonucleotide primer (CODEHOP) strategy, in order to identify genetic variability, possibly linked to the functional α-AI differences. The cultivars Pisu Grogu and Fasolu α-AI share more molecular characteristics with isoform α-AI-4 rather than with isoform α-AI-1.

This study revealed that the Sardinian cultivar called Nieddone is of particular interest for its inhibitory activity on digestive enzymes and for the absence of hemagglutination power on human red cells. Its purified inhibitor has a tetrameric structure with a molecular

weight of about 42 kDa, an interesting stability to high temperatures, and a relevant durability of inhibitory activity over the time.

The effectiveness of the purified α-AI against pig, human and insect α-amylase enzyme highlights the potential of this protein in the obesity and diabetes treatment and in the management of insect pests.

A future goal will be to complete cDNA sequencing of all these cultivars to increase understanding of the primary structure of these proteins, improving the mechanistic characterization of this protein-protein interaction, in order to valorise the Sardinian biodiversity and increase the commercial value of some cultivar such as Nieddone.

A possible strategy could be the nano-formulation of the most promising species to improve their bio-pharmaceutical properties, such as resistance to proteolytic degradation in both mammalian and insect gastrointestinal tract.

However, before developing commercial application of a cultivar, a crucial point to be investigated concerns the feasibility of extensive cultivation, especially in the case of the Nieddone cultivar. In fact, it must take into consideration that these cultivars currently are only cultivated in very small plot of lands, present in very limited spatial regions.

Therefore, it will be necessary to deepen the agronomic aspects related to the plant needs in terms of water supply, sun exposure and light, soil type, nutrient and fertilizer. These pieces of information will be useful in understanding whether these cultivars can be cultivated on a large scale, in greenhouses or on open ground in Sardinia or other regions. Even though the common bean is mainly self-pollinated, natural hybridization in both and wild cultivated populations is reported [235]. Therefore, it is necessary to take precautions for maintain genetic integrity.

At this regard, it will be necessary to involve not only botanists, but also farmers and entrepreneurs interested in cultivating these cultivars for use in the production of food supplements and for the commercialisation. In addition, resistance to local pathogens and insects will have to be studied and possible treatments against these agents evaluated.

On the whole, this study has broadened the knowledge about this class of proteins and these 9 poorly studied common bean cultivars, fuelling the valorisation of Sardinian biodiversity.

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Ringraziamenti

E come tutte le cose belle che prima o poi finiscono, volge al termine anche questo splendido percorso. Ebbene sì, perché per me questi tre anni sono passati così velocemente…quasi da non accorgermene.

Se questo è avvenuto è stato solo grazie alle persone che mi hanno circondato durante questo periodo.

Il primo grazie va alla Prof.ssa Olianas per essere stata la prima a credere in me e per avermi fatto conoscere il meraviglioso mondo della Biochimica.

Grazie alla Prof.ssa Padiglia e alla Dott.ssa Cannea per avermi accolto nei vostri laboratori, per tutte le frasi di affetto e di sostegno che mi avete sempre riservato e per tutto il tempo che mi avete dedicato.

Grazie alla Prof.ssa Dettori per i suoi sorrisi e per la disponibilità. Grazie a Prof. Solari che so aver sempre vegliato su di me anche a distanza.

Grazie a Sonia per tutte le giornate trascorse insieme in laboratorio. Mia compagna di esperimenti e di risate. Senza di te il percorso sarebbe stato sicuramente più noioso.

Grazie alla Prof.ssa Viviana Cristiglio per avermi accolta come una di famiglia a Grenoble e grazie Luca e Giuseppe per avermi fatta sentire a casa.

Grazie alla mia famiglia.

Grazie agli amici di una vita e a quelli con cui ho condiviso questo percorso. Mariano, Cinzi e Giacomo non vi ringrazierò mai abbastanza.

Grazie a te che hai deciso di esserci e di restare.

Grazie anche a chi ora non c'è più ma che mi ha insegnato la forza e cosa significhi essere una guerriera.

Un grazie speciale a Puppinis perché non c'è nulla di meglio di tornare a casa dopo una lunga giornata di lavoro e trovare un naso umido e uno scovolino scodinzolante…

Ed infine devo ringraziare voi, che avete permesso tutto questo. Grazie Prof. Zucca e Prof. Rescigno.

Siete stati la mia famiglia, i miei amici, un faro per la mia crescita personale e professionale. Avete avuto una pazienza infinita e mi avete donato veramente tanto; spero un giorno di poter ricambiare anche solo in minima parte quello che mi avete dato.

Paolo, sei stato molto più che un Professore, un amico, un compagno di viaggi, un tutor esemplare. Mi hai insegnato che è sempre bene provare a fare le cose, a non

arrendermi mai, ad andare avanti. Mi mancherà molto non stressarti più a tutte le ore del giorno e sentire le tue parole magiche "Non ti preoccupare…!"

Anto, sei stato come un papà… Grazie per tutte le cose che mi hai insegnato, per il sarcasmo e l'umorismo di tutti i giorni. Mi mancheranno le colazioni al bar (soprattutto quelle offerte da Paolo), i tuoi preziosissimi consigli, la tua gentilezza ed eleganza.

Hemingway diceva che "Il modo migliore per scoprire se ci si può fidare di qualcuno è di dargli fiducia" e voi lo avete fatto con me. Spero di non averla tradita mai.

Questo lavoro è tutto per voi….