STORAGE GLOBULIN AND ASPARTIC PROTEINASE FROM UNGERMINATED CACAO SEEDS: KINETICS AND MECHANISM OF *IN VITRO* PROTEOLYSIS

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Din semințele negerminate de cacao a fost obținută proteina de rezervă 7S globulina şi proteinaza aspartică purificată prin cromatografia de afinitate pe pepstatin A imobilizat. Ca şi celelalte proteinaze aspartice din plante, proteinaza aspartică din cacao este un heterodimer al lanțului polipeptidic mare şi mic (34 kDa şi 16 kDa, respectiv). Hidroliza globulinei 7S cu proteinaza aspartică a fost caracterizată prin SDS-electroforeză şi prin determinarea proteinei reziduale şi a azotului aminic în hidrolizat. Au fost detectate trei reacții independente de ordinul pseudo-întîi: proteoliza rapidă nelimitată (cooperativă) a parții nestabile (aproximativ 20%) din globulina 7S sumară, care rapid se elimină la începutul incubației, proteoliza cooperativă lentă a reziduului globulunei 7S stabile şi proteoliza limitată comparativ lentă, care constă în scindarea singulară a subunităților globulinei 7S. Rezultatele obținute descriu degradarea globulinei 7S catalizată de proteinaza aspartică la fermentarea boabelor de cacao.

Proteins and proteolytic enzymes in cacao (*Theobroma cacao* L.) seeds and proteolysis during cocoa bean fermentation have been studied with respect to cocoa quality [1-5]. 7S globulin (vicilin) was found to be one of the two storage proteins in cacao seeds [1,4,6,7]. The mature cacao 7S globulin is derived from a 67 kD precursor [6,7], which is cleaved to form a 45-47 kD chain [4,6,8] characteristic of many ordinary vicilins [9]. Two chains of lower molecular mass (31 kD and 16 kD) found to be the other constituents of cacao 7S globulin molecule [4,6] probably resulted from an additional posttrsnslational cleavage some of the 45-47 kD chains. Like other 7S globulins, the cacao 7S globulin molecule seems to be a trimer assembled by the random joining of several non-identical but homologous 45-47 kD and (31+16) kD chains.

Ungerminated cacao seeds are abundant in aspartic proteinase (CAP) accounting for about 98 per cent of the total endoproteolytic activity at pH 4.5 [10]. The enzyme is responsible for 7S globulin breakdown during both fermentation of cocoa beans [1] and autolysis of crude extracts from acetone powder of ungerminated cacao cotyledons [10]. The CAP represented by two closely similar sequences TcAp1 (emb|CAC86003) and TcAp2 (emb|CAC86004) [11] belongs to a highly conserved family of plant aspartic proteinases [12].

Physiological role of CAP in cacao seeds remains unclear. In spite of a high activity of CAP both in ungerminated seeds and seedling cotyledons, the *in vivo* degradation of the 7S globulin is delayed for about 10 days [1]. Hence, a mechanism of 7S globulin protection against CAP activity should exist. It should be noted that in the experiments cited above [1,10] the CAP has not been purified and might contain admixtures of other proteinases. As it was shown recently, a papain-like proteinase abundant in *Phaseolus vulgaris* seedling cotyledons acquires the ability to catalyze unlimited degradation of 7S globulin only after its limited proteolysis carried out by another endogenous proteinase, namely Asn-specific legumain [13]. Therefore, the ability of CAP to degrade directly the native (non-modified proteolytically) 7S globulin is probable but not proven.

Purification both of CAP and 7S globulin from ungerminated cacao seeds was carried out in this investigation. The action of pure CAP on native 7S globulin was followed. Mixed-type limited and unlimited (cooperative) proteolyses occurring in parallel was observed and its kinetics was described according to an approach developed earlier [14].

Materials and Methods

Cacao (*Theobroma cacao* L.) seeds from genetically undefined Forastero pods provided by CRIG, Tafo, Ghana (Amelonado type) and by MARDI, Malaysia (mixed hybrids) were extracted from ripe, healthy pods about 3 to 5 days after harvest. Acetone dry powder was prepared from cotyledons as described previously [3] by shock freezing, lyophylization, extraction of fat by petroleum benzene followed by complete elimination of polyphenols and purines using ice-cold acetone and mixtures of acetone/water 80:20 (by volume) containning 0.1 % (by volume) thioglycollic acid.

Purification of CAP. All procedures were carried out at 4°C or in ice in presence of 0.02% NaN_3. The enzyme was extracted from 10 g of acetone powder by 200 ml of 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2.0 mM EDTA (Buffer 1). The supernatant was loaded into a column (2.6 x 18 cm) of Phenyl Sepharose CL-4B equilibrated with Buffer 1. After washing of the column with the same buffer, the enzyme was eluted by water, adjusted to pH 4.3 by Buffer 2 (0.1 acetate pH 4.0, 0.5 M NaCl) and loaded into a column (bed volume 2.0 ml) of Pepstatin A agarose (Sigma) equilibrated with Buffer 2. Binding of CAP to the resin was allowed overnight by recycling the enzyme solution through the column. After removal of the non-adsorbed fraction, the column was washed with 0.1 M Tris-HCl, pH 7.5, 0.5 M NaCl. CAP was eluted with 0.1 M bicarbonate, pH 10.0, 0.5 M NaCl. The enzyme solution was adjusted to pH 4.3 with Buffer 2 and re-chromatographed.

Assay of CAP activity. The activity was assayed at pH 4.5 (0.15 M citrate/phosphate buffer adjusted by NaCl to ionic strength 0.5), 30°C, using bovine serum albumin as a substrate (final concentration 1%). The reaction was stopped by addition of trichloroacetic acid (final concentration 4%). Amino nitrogen of the supernatant was determined as described earlier [14] using 2,4,6-trinitrobenzenesulphonic acid. The enzyme activity was expressed in international units IU (μmoles per min of amino groups released). The protein in the enzyme preparations was determined according to Bradford [15} using bovine serum albumin as a standard.

Purification of 7S globulin. All the solutions contained 0.02% NaN_3. The chromatographic procedures were carried out at room temperature. The acetone dry powder (10 g) was extracted three times in an ice bath by 200 ml of buffer 1 containing proteinase inhibitors (15 μM pepstatin A, 5 mM iodoacetic acid and 5 mM phenylmethylsulfonylfluoride). The supernatants containing protein contaminants were discarded. The pellet was extracted twice by 100 ml of 0.2 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. The combined extracts were adjusted to ionic strength 4.0 by addition of solid $(NH_4)_2SO_4$ and loaded into a column (2.6 x 18 cm) of Phenyl Sepharose CL-4B equilibrated with 1.33 M $(NH_4)_2SO_4$. The column was washed by 1.33 M $(NH_4)_2SO_4$ adjusted to pH 8.0 by NH_4OH, and the 7S globulin was eluted with 0.17 M $(NH_4)_2SO_4$ adjusted to pH 8.0. The globulin solution was concentrated by ultrafiltration, dialyzed against 10 mM Tris-HCl buffer, pH 8.0, and subjected to gel filtration on a column (2.6 x 80 cm) of Sephacryl S-300 equilibrated with the same buffer. The purified 7S globulin was concentrated by ultrafiltration.

Polyacrylamide gel electrophoresis. SDS-electrophoresis (SDS-PAGE) was carried out on 15% gels according to Laemmli [16] using Sigma molecular mass standards. Image analysis software (QuantiGel Corp., U.S.A) was used for quantitation of polypeptides.

Kinetics of hydrolysis of 7S globulin by CAP. According to Shutov *et a*l. [14], unlimited (cooperative) proteolysis occurs as a pseudo first order reaction described by two equations. First equation, $P(t) = P_0e^{-kt}$, where $P(t)$ and P_0 are the residual and initial concentrations (by mass) of the protein substrate, respectively, k is the rate constant, and t is duration of proteolysis. Second equation, $N(t) = N(1-e^{-kt})$, where N is the molar concentration of peptides per one molecule of protein substrate that are formed by exhaustive cooperative proteolysis.

7S globulin and CAP solutions in 0.15 M citrate/phosphate buffer, pH 4.5, adjusted by NaCl to ionic strength 0.5, 0.02 % NaN_3, were mixed and incubated at 30°C (6.0 ml of incubation mixture containing 18 mg of 7S globulin and 0.1 IU of CAP). In aliquots taken after the indicated time periods, the reaction was stopped by addition of trichloroacetic acid up to a final concentration of 6% (w/v). The precipitated protein was determined according to Bradford [15]. Amino nitrogen in the supernatant was determined as described earlier [14] using 2,4,6-trinitrobenzenesulphonic acid. The data obtained were expressed as P_0 taken as unit and respective $P(t)$, and as $N(t)$ [14].

Detection of glycosylated polypeptides. Polypeptides separated by SDS-PAGE were analyzed for carbohydrate side chains essentially as described by O'Shanessy *et al*. [17] by use of the DIG glycan detection kit (Boehringer, Mannheim, Germany).

Results

Purification of CAP. Phenyl Sepharose chromatography is a convenient way of preliminary purification of CAP (Table). The 1.3-1.6-fold increase of the total proteolytic activity was observed in different experiments after affinity chromatography. Possibly, it is resulted from removal of an inhibitory factor (either specific or non-specific). SDS-PAGE revealed several bands of contaminating proteins completely eliminated by re-chromatography. According to SDS-PAGE of the final enzyme preparation (Fig.1), the CAP is a hetero-dimer of 33.9 kD and 15.7 kD chains. This structure is characteristic most of plant aspartic proteinases [12] including the enzyme from barley grains, whose 3 D structure is available (pdb|1QDM).

Table

Purification of CAP

Purification step	Volume, *ml*	Protein, *mg*	Activity *IU*	Specific activity, *IU/mg*	Yield, *%*	Purification - *fold*
Crude extract	175	444	11.9	0.0268	100	1
Phenyl Sepharose	27.0	61.0	3.76	0.0616	31.6	2.30
Pepstatin Sepharose:						
First run	8.1	2.20	4.82	2.19	40.5	81.8
Second run	6.6	1.69	5.86	3.47	49.2	129

In the barley enzyme, 32 kD and 16 kD chains are linked by a disulfide bridge [18] absent in the case of CAP (Fig.1). This distinction might be explained in a following way (Fig.2). Plant aspartic proteinases are synthesized as precursors containing internal plant specific insert (PSI) either totally or partially posttranslationally removed generating long N-terminal and short C-terminal polypeptides of mature enzymes [12}. All six disulfide bonds in the precursors of plant aspartic proteinases are located within limits of long/short polypeptides and PSI. The latter contains Cys residues in its C-/N-termini, which are involved in formation of the disulfide bond C289-C383 (pdb|1QDM). Therefore, only incomplete removal of the PSI might explain disulfide bridging of the polypeptides in mature molecule of the barley enzyme [18]. And *vice versa*, the absence of a disulfide bond between CAP chains implies either complete or at least extended posttranslational removal of the PSI.

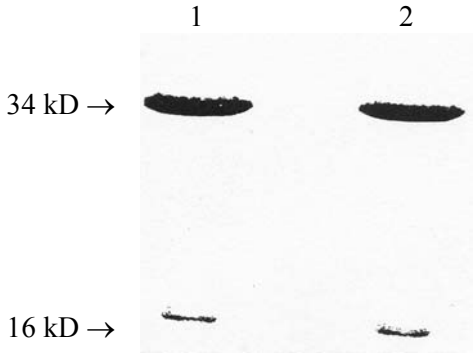

Fig.1. SDS-PAGE of purified CAP in reducing (1) and non-reducing (2) conditions.
A slight shift of the position of both the bands in non-reducing conditions indicates presence of intra-chain (rather than inter-chain) disulfide bonds in the CAP dimer.

Purification of 7S globulin. Phenyl Sepharose chromatography revealed two 7S globulin fractions eluted with 0.17 M $(NH_4)_2SO_4$ and water, respectively. Enhanced surface hydrophobicity of the water-eluted fraction might be resulted from partial denaturing of the 7S globulin. This fraction thereunto containing most of contaminating proteins was discarded. The residual contaminating proteins were removed by gel filtration at low ionic strength (≤ 0.01) resulting in purity of 7S globulin (see Fig.3, zero time). During gel filtration, the 7S globulin is eluted as a trimer or as a dimer of trimers at a high and low ionic strength, respectively (data not shown). Dimerization of trimers at low salt concentration also is characteristic of soybean 7S globulin [19].

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                                          _________S-S__________
     Long chain                          |                      |       Short chain
1QDM AIADSGTSLLAGPTAIITEINEKIGAAGVVSQECKTIVSQYG//TQDLILDYVNQLCNRLPSPMGESAVDCGSLG
CAP  AIADSGTSLITGPTAIIAQVNHAIGASGVVSQECKTVVSQYG//TQERILEYINELCDRLPSPMGESAVDCSSLS
     *********  ******   *  *** ********* *****  **  *  * * ** ************ **
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Fig.2. Possible explanation for the absence/presence of inter-chain disulfide bridge in mature plant aspartic proteinases from barley grains (pdb|1QDM) and cacao seeds (CAP).
Underlined characters indicate position of a 103-residue plant specific insert (PSI). Asterisks denote sequence identities. Non-cleaved off C-/N-terminal segments of the PSI in the barley proteinase, which contain Cys residues, are suggested to be responsible for the retention of a disulfide bridge between long and short polypeptides in the mature enzyme.

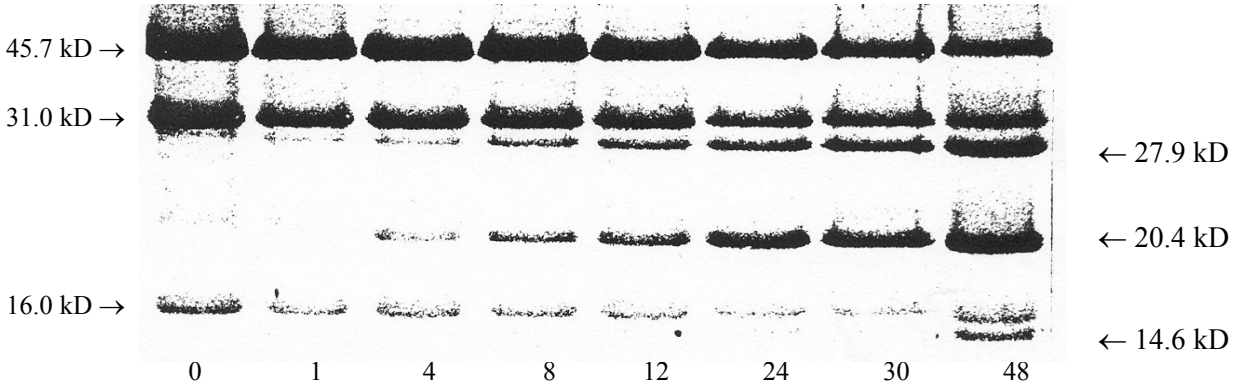

Fig.3. Limited proteolysis of cacao 7S globulin by CAP followed by SDS-PAGE of the residual protein. The numbers below lanes correspond to the duration of proteolysis (hrs). The bands of intact 7S globulin chains (left side) and their fragments (right side) are shown according to their apparent molecular masses calculated using co-running molecular mass standards.

A hypothetical scheme of 7S globulin limited proteolysis catalyzed by CAP. Three polypeptide chains of apparent molecular masses 45.7 kD, 31.0 kD and 16.0 kD as the constituents of the intact mature 7S globulin were displayed by SDS-PAGE (Fig.3, zero time). Equimolarity of 31 kD and 16.0 kD chains (molar ratio 1:0.96) was found from SDS-PAGE data in accordance with the presumed origin of both from posttranslationally cleaved 45.7 kD chain [4,6]. According to Spencer and Hodge [6], the 31 kD chain corresponds to the N-terminal part of the initial non-cleaved (31+16) polypeptide.

SDS-PAGE revealed three polypeptide fragments (27.9 kD, 20.4 kD and 14.6 kD) formed due to limited proteolysis during the action of CAP on 7S globulin (Fig.3). The origin of these fragments shown in a hypothetical scheme (Fig.4) can be deduced on the basis of following data. The 20.4 kD fragment was found glycosylated (data not shown). A single potential glycosylation site in cacao 7S globulin sequence (prf|1905429A) is located at the N-terminus of the mature subunits. Thus, the 20.4 kD fragment (as well as the 31 kD chain [6]) belongs to the N-terminal part of the subunit suggesting formation the 20.4 kD fragment from both 45.7 kD and 31 kD chains.

Kinetics of mixed-type proteolysis of 7S globulin by CAP. Linearity of the plot *lnP vs t* was observed after 1.5-2 hrs of the reaction during all the subsequent period studied up to the breakdown of at least 80 % of the initial protein (Fig.5A). Hence, the decrease in *P* value during the linear second stage of proteolysis is the result only of the cooperative process in spite of limited proteolysis that occurs in parallel (Fig.3,4). The preservation of linearity of the plot during mixed-type (cooperative + limited) proteolysis of the 7S globulin can be regarded as an indication of (i) the constancy of the molecular mass of non-dissociated protein substrate at all the linear region of the plot *lnP vs t* irrespective of cleavage of its chains; (ii) the equality of the rate constants of cooperative proteolysis of the intact 7S globulin and of that modified by limited proteolysis, which are both present in the reaction medium (Fig.3).

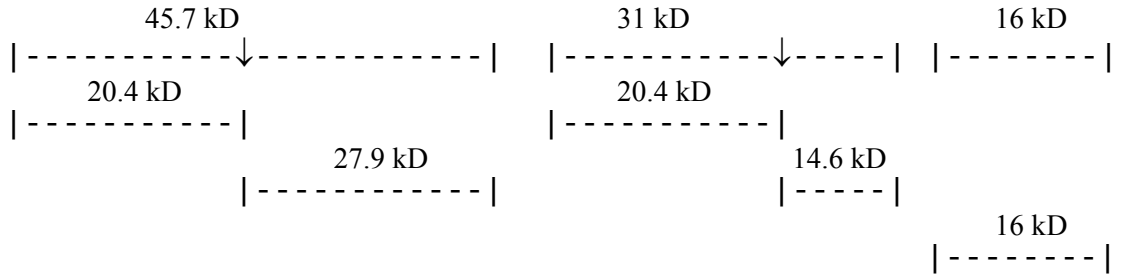

Fig.4. A hypothetical scheme of limited proteolysis of cacao 7S globulin chains carried out by CAP. Intact 7S chains and their fragments are designated in accordance with their apparent molecular masses determined by SDS-PAGE. Identical single cleavage points in 45.7 kD and (31+16) kD subunits might explain formation of all the three polypeptide fragments revealed by SDS-PAGE (Fig.3). According to kinetic analysis (Fig.5) limited proteolysis of the 7S globulin is not accompanied with detachment of any low-molecular mass peptides.

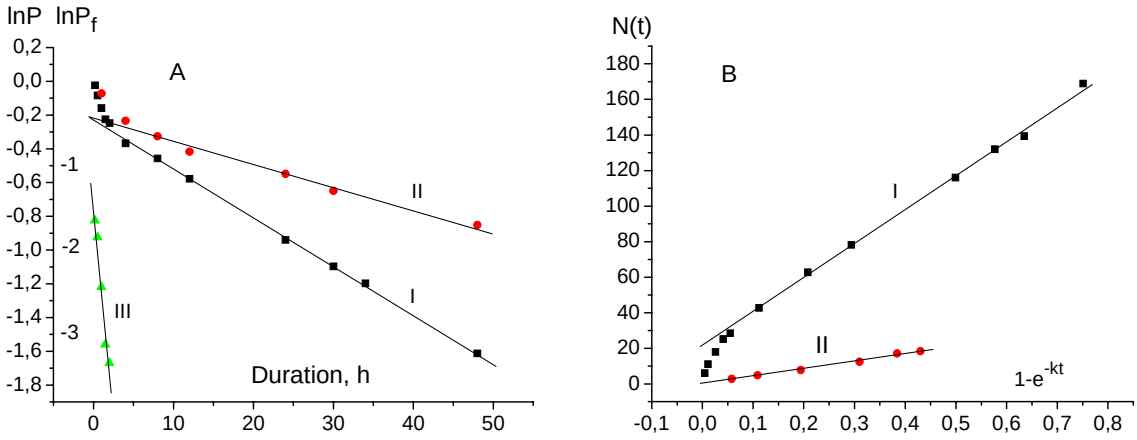

Fig.5. Kinetics of hydrolysis of cacao 7S globulin by CAP.
A, kinetic curves describing cooperative and limited proteolyses. I, the plot *lnP vs t*. The concentration (by mass) of the total residual protein *P(t)* is expressed as a fraction of its initial concentration P_0. II, the plot lnP_f *vs t*. The concentration (by mass) of quickly hydrolyzed residual protein P_f is expressed as a fraction of the initial concentration of the total protein P_0. The $P_f(t)$ values calculated from the plot I were taken as differences between *P(t)* values, experimental and corresponding to the linear part of the plot I, respectively. III, the plot *lnI vs t*, where *I* is a relative intensity of the sum of 45.7 kD and 31 kD bands. B, current molar concentration *N(t)* of peptides formed by cooperative proteolysis from one mole of protein substrate plotted *vs* $(1\text{-}e^{kt})$ (see Materials and Methods). The *N(t)* values were calculated for total 7S globulin *P*, rate constant k_s (I) and for its unstable part P_f, rate constant k_f, (II). Concentrations of peptides derived from the unstable part of the 7S globulin were taken as an absolute difference between *N(t)* values, experimental and corresponding to the linear part of the plot I, respectively.
Note: the relationship II was plotted on a twofold scaled down abscissa.

Curvilinear initial part of the plot *lnP vs t* probably results from the summation of slow and fast pseudo first order reactions (Fig.5A) of sharply different rate constants k_s and k_f (k_s/k_f = 1:34). No indications were obtained for the occurrence of a quick limited proteolysis during the first two hrs of the reaction (Fig.3). Therefore, both the quick and slow components of the proteolysis occur via cooperative mechanism. This implies that the initial 7S globulin preparation consists of slowly and quickly hydrolyzed parts (P_s and P_f, respectively). The P_s value calculated by extrapolation of the linear part of the plot *lnP vs t* to zero time is equal to 0.79. Probably, the existence of two 7S globulins different from each other in susceptibility to cooperative proteolysis is a consequence of its heterogeneity. Recently, we observed that the susceptibility to cooperative proteolysis of *Ph. vulgaris* 7S globulin depends on subunit composition of its trimers [20].

Along the action of CAP, limited proteolysis consists in a single cleavage of initial 45.7 kD and 31 kD chains. Therefore, gradually decreased values of relative intensity of the sum of these bands *I* can be used to describe kinetics of 7S globulin limited proteolysis that should be a pseudo-first order reaction [14]. Indeed, the plot *lnI vs t* is linear during the second stage of proteolysis (Fig.5B). The corresponding rate constant k_i is 2.2 times lower than the k_s value. This conforms to the detection of a relatively large quantity of the 7S globulin chains, which are still intact even after breakdown via cooperative mechanism of the major part of the initial protein (Fig.5A).

At the first stage of proteolysis, because of the great difference in the rate constants k_i and k_f (k_i/k_f = 1:70), the concentration P_f of the unstable part of the 7S globulin declines rapidly by cooperative process, whereas its limited proteolysis is negligible. Therefore, the relative concentration *I* of the intact protein decreases rapidly at the first stage (Fig. 5 A) because of quick elimination of non-cleaved 7S globulin chains via cooperative process. The extrapolation of the linear part of the plot III to zero time leads to apparent value I_s coincided with relative concentration P_s of the stable part of the 7S globulin.

To describe kinetics of formation of peptides derived from exhaustive cooperative proteolysis of stable P_s and unstable P_f parts of the 7S globulin we used rate constants k_s and k_f, respectively, calculated from Fig.5A. The resulted plots *N(t) vs* $(1\text{-}e^{kt})$ revealed linearity at the second slow and at the first quick stages of proteolysis (Fig.5 B, the plots I and II, respectively).

Discussion

Utmost precautions were taken to prevent *in vitro* proteolytic modification of 7S globulin during preparation of dry acetone powder from cacao cotyledons and during isolation of the 7S globulin. A portion of the 7S globulin of heightened surface hydrophobicity presumably corresponding to denatured protein was eliminated by Phenyl Sepharose chromatography. Therefore, it is likely that at least the relatively stable part (79%) of 7S globulin preparation studied is really native. It can be directly *in vitro* degraded by CAP preparation containing no admixtures. Thus, the mentioned above *in vivo* protection of the 7S globulin against CAP activity might be due to their different cellular compartmentation (or even sub-compartmentation, see [21] for references).

Sequence information on cacao 7S globulin subunits is scanty and sometimes even confusing. Several incomplete sequences reveal 99-100% identity to two complete sequences currently available (emb|CAA44493 and prf|1905429A). The complete sequences also reveal 100% identity in 502-residue region, which covers signal peptide, the N-terminal extension posttranslationally removed, and almost total sequence of the mature protein. The region that probably form the C-terminal α-helix domain in prf|1905429A is completely absent in emb|CAA44493. Thus, the latter cannot be involved in formation of 7S trimer characteristic of any genuine vicilin. The source of this confusion is a mystery, and one can conclude that only a single sequence of cacao 7S globulin (prf|1905429A) really is available.

Most probably, subunit structure of cacao 7S globulin is as complicated as it was shown for other seed vicilins (*i.e.* composed of homologous but non-identical subunits [9]). Possibly, additional posstranslational cleavage that forms 31 kD/16 kD polypeptide pair occurs in a subunit containing specific cleavage site absent in other subunits; this phenomenon is known for some other storage globulins (see [21] for references). Similarly, the presence of the stable and unstable parts in cacao the 7S globulin preparation might be due to heterogeneity of its natural trimers. As it was mentioned above, we found that the stability of *Ph. vulgaris* 7S globulin depends on subunit composition of its trimers [20].

Degradation of seed storage globulins by endogenous papain-like proteinases and trypsin starts with quick limited proteolysis, which consists in cleavage of specifically extended hydrophilic inserts of a high susceptibility to proteolytic attack [21]. These inserts easily recognizable in cacao 7S globulin (the inter-domain linker and the EF loop in the C-terminal module) are hydrophilic as well. They probably are inaccessible to CAP, which like other aspartic proteinases of the A1 family [12] should preferentially cleave peptide bonds between bulky hydrophobic side chains. Therefore, limited proteolysis of the 7S globulin by CAP is restricted to a single cleavage (roughly, at the beginning of the α-helix domain of the N-terminal module). It seems believable that the susceptibility of the region of this cleavage, which probably belongs to structurally ordered elements, should be rather low. Thus, the limited proteolysis of the 7S globulin occurs slowly in parallel to protein breakdown via cooperative mechanism.

The linearity of both the plots $P(t) = P_0e^{-kt}$ and $N(t) = N(1-e^{-kt})$ at the second stage of the reaction (after exhaustion of the unstable part) implies constancy of the summary molecular mass of the residual protein irrespective on cleavage of initial polypeptides. This circumstance allowed describing the kinetics of limited proteolysis that occurred as a pseudo-first order reaction as well as the cooperative process.

In summary, the described data shows the pattern of 7S globulin degradation catalyzed by CAP during cacao bean fermentation, which brings about the formation of cocoa flavor precursors [1,5,11].

References:

1. Biehl B., Wewetzer C., Passern D. Vacualar (storage) proteins in cocoa seeds and their degradation during germination and fermentation // J. Sci. Food Agric. - 1982. - Vol.33. - P.1291-1304.
2. Biehl. B., Brunner E., Passern D., Quesnel V.C., Adomako D. Acidification, proteolysis and flavor potential in fermenting cocoa beans // J. Sci. Food Agric. - 1985. - Vol.36. - P.583-589.
3. Kirshoff P.M., Biehl B., Crone G. Peculiarity of the accumulation of free amino acids during cocoa fermentation // Food Chem. - 1989. - Vol.31. - P.295-311.
4. Voigt J., Biehl B., Kamaruddin S.W. The major seed proteins of *Theobroma cacao* L. // Food Chem. - 1993. - Vol.47. - P.145-151.
5. Voigt J., Biehl B., Heinrichs H., Kamaruddin S.W., Gaim Marsoner G., Hugi A. *In vitro* formation of cocoa specific aroma precursors: aroma-related peptides generated from cocoa-seed protein by co-operation of an aspartic endoprotease and a carboxypeptidase // Food Chem. - 1994. - Vol.49. - P.173-180.

6. Spencer M.E., Hodge R. Cloning and sequencing of a cDNA encoding the major storage proteins of *Theobroma cacao*. Identification of the proteins as members of the vicilin class of storage proteins // Planta. - 1992. - Vol.186. - P.567-576.
7. McHenry L., Fritz P.J. Comparison of the structure and nucleotide sequences of vicilin genes of ccocoa and cotton raise questions about vicilin evolution // Plant Mol. Biol. - 1992. - Vol.18. - P.1173-1176.
8. Yavuz M.O., Ashton S.M., Deakin E.D., Spencer M.E., Sudbery P.E. Expression of the major bean proteins from *Theobroma cacao* (cocoa) in the yeasts *Hansenuls polymorpha* and *Saccharomyces cerevisae* // J. Biotechnol. - 1996. - Vol.18. - P.43-54.
9. Dunwell M.J. Structure, function, and evolution of vicilin and legumin seed storage proteins // In: Polysaccharides and polyamides in the food industry: properties, production, and patents (A. Steinbüchel, S.K.Rhee, eds.). WILEY-VCH Verlag, Weinheim. - 2005. - Vol.2. - P.707-738.
10. Biehl B., Heinrichs H., Ziegeler-Berghaisen H., Hammoor M., Senyuk V. The proteases of ungerminated cocoa seeds and their role in the fermentation process // Angew. Bot. - 1993. - Vol.67. - P.59-65.
11. Laloi M., McCarthy J., Morandi O., Gysler C., Bucheli P. Molecular and biochemical characterization of two aspartic proteinases TcAP1 and NcAP2 from *Theobroma cacao* seeds // Planta. - 2002. - Vol.215. - P.754-762.
12. Simőes I., Faro C. (2004) Structure and function of plant aspartic proteinases // Eur. J. Biochem. - 2004. - Vol.271. - P.2067-2075.
13. Zakharov A.D., Carchilan M., Stepurina T.G., Rotari V.I., Wilson K.A., Vaintraub I.A. A comparative study of the role of the major proteinases in the degradation of storage proteins in germinating common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* (L.) Merrill) seeds // J. Exp. Bot. - 2004. - Vol.55. - P.2241-2249.
14. Shutov A.D., Pineda J., Senyuk V.I.., Reva V.A., Vaintraub I.A. Action of trypsin on glicinin: Mixed-type proteolysis and its kinetics. Molecular mass of glycinine-T // Eur. J. Biochem. - 1991. - Vol.199 - P.539-543.
15. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding // Anal. Biochem. - 1976. - Vol.72. - P.248-254.
16. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4 // Nature. - 1970. - Vol.227. - P.680-685.
17. O'Shanessy D.J., Voorstad P.J., Quarles R.H. Quantitation of glycoproteins on electroblots using the biotin-streptavadin complex // Anal. Biochem. - 1987. - Vol.163. - P.204-209.
18. Sarkkinen P., Kalkinen N., Tilgmann C., Siuro J., Kervinen J., Mikola L. Aspartic proteinase from barley grains is related to mammalian lysosomal cathepsin D // Planta. - 1992. - Vol.186. - P.317-423.
19. Thanh V.H., Shibasaki K. Major proteins of soubean seeds. Subunit structure of β-conglycinin // J. Agric. Food Chem. - 1978. - Vol.26 - P.692-695.
20. Rudakova A., Rudakov S., Lapteva N., Morari D., Stepurina T., Rotari V., Kakhovskaya I., Wilson K., Fukuda T., Utsumi S., Shutov A. *In vivo* and *in vitro* limited proteolysis of phaseolin: facts, suggestions and problems // Anale stiinţifice ale USM, seria Ştiinţe chimico-biologice. - Chişinău: CEP USM. - 2007.
21. Shutov A.D., Blattner F.R., Bäumlein H., Müntz K. Storage and mobilization as antagonistic functional constraints on seed storage globulin evolution // J. Exp. Bot. - 2003. - Vol.54. - P.1645-1654.

Acknowlegement. *The authors are grateful to the Cocoa Research Institute of Ghana (CRIG) and the Malaysian Agricultural Research and Development Institute (MARDI), Cocoa and Coconut Division for providing freshly harvested cocoa pods.*

Prezentat la 30.07.2007