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Raw Starch-digesting Amylase is Comprised of two Distinct Domains of Catalytic and Substrate-Adsorbable Domain: Role of the C-Terminal Region in Raw-Starch-Binding

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Summary

Raw starch-digesting amylase (BF-2A, M.W. 93,000 Da) from *Bacillus circulans* F-2 was converted to two components during digestion with subtilisin. Two components were separated and designated as BF-2A' (63,000 Da) and BF-2B (30,000 Da), respectively. BF-2A' exhibited the same hydrolysis curve for soluble starch as the original amylase (BF-2A). Moreover, the catalytic activities of original and modified enzymes were indistinguishable in K_m, Vmax for, and in their specific activity for soluble starch hydrolysis. However, its adsorbability and digestibility on raw starch was greatly decreased. Furthermore, the enzymatic action pattern on soluble starch was greatly different from that of the BF-2A. A smaller peptide (BF-2B) showed adsorbability onto raw starch. By these results, it is suggested that the larger peptide (BF-2A') has a region responsible for the expression of the enzyme activity to hydrolyze soluble substrate, and the smaller peptide (BF-2B) plays a role on raw starch adsorption. A similar phenomenon is observed during limited proteinase K, thermolysin, and endopeptidase Glu-C proteolysis of the enzyme. Fragments resulting from proteolysis were characterized by immunoblotting with anti-RSDA. The proteolytic patterns resulting from proteinase K and subtilisin were the same, producing 63- and 30-kDa fragments. Similar patterns were obtained with endopeptidase Glu-C or thermolysin. All proteolytic digests contained a common, major 63-kDa fragment. Inactivation of RSDA activity results from splitting off the C-terminal domain. Hence, it seems probable that the protease sensitive locus is in a hinge region susceptible to cleavage. Extracellular enzymes immunoreactive toward anti-RSDA were detected through whole bacterial cultivation. Proteins of sizes 93-, 75-, 63-, 55-, 38-, and 31-kDa were immunologically identical to RSDA. Of these, the 75-kDa and 63-kDa proteins corrrespond to the major products of proteolysis with Glu-C and thermolysin. These results postulated that enzyme heterogeneity of the raw starch-hydrolysis system might arise from the endogeneous proteolytic activity of the bacterium. Truncated forms of *rsda*, in which the gene sequence encoding the conserved domain had been deleted, directed the synthesis of a functional amylase that did not bind to raw starch. This indicates that the conserved region of RSDA constitutes a raw starch-binding domain, which is distinct from the active centre. The possible role of this substrate-binding region is discussed.

Introduction

B. circulans F-2 produces large quantities of carbohydrate-utilizing enzymes with different specificities. Unlike other amylase-producing microorganisms, this bacterium produces amylase only

when raw starch granules are used as a carbon source. Raw potato starch was found to result in the highest amylase production. Raw potato starch induced about 5 times more amylase production than raw corn starch[1]. Raw starch-granule-hydrolytic system exists in the bacterium in multiple forms, consisting of at least 6 different kinds of proteins [2]. Of these proteins, BF-2A (93kDa), which is the most abundant, has been best studied with regard to its structural properties. The gene coding for this enzyme has been cloned[3] and a special region was found in the deduced polypeptide chain that was rich in serine and threonine.

With these specific charateristics of this amylase, to know the raw starch digestibility, when this amylase was digested with subtilisin, it was converted to two components (BF-2A' and BF-2B). These assays showed that BF-2A' was a gelatinized starch-digestible amylase and BF-2B was a adsorption peptide. BF-2A' had a decreased ability to adsorb onto raw starch and had a little ability to digest raw starch. These results showed that the modified amylase (BF-2A') had a essential region for soluble starch-digestion and a hydrolytic domain of the original amylase.

Subtilisin and proteinase K cleave BF-2A at the same site, close to the C-terminal domain. Cleaved, purified BF-2A shows no alteration of hydrolytic properties against soluble starch. These datas suggest that inactivation is probably due to a loss of ability of the C-terminal domain to bind to raw substrate. The C-terminal domain may form part of the binding site for raw substrate [4-6]. The extracellular bacterial fraction immunoreacted with anti-RSDA containing 6 different proteins, and the molecular weights of these proteins were similar to those of Glu-C and thermolysin-digests. The results indicate that the multiplicity of extracellular enzymes responsible for effective hydrolysis of raw starch granules may be caused by endogeneous proteolysis in the extracellular environment of the bacterium.

The functional role of the carboxyl-terminal sequences of the RSDA is investigated using clones encoding either the entire *rsda* or the truncated forms of *rsda*, which has the C-terminal sequences deleted. The comparison of the enzymatic properties of the the enzymes reveals that the C-terminal part of the enzyme is involved in the ability of this enzyme to bind raw starch.

Materials and Methods

Microorganism, cultivation, adsorption of amylase onto raw starch, assay of enzyme activity and digestion of raw starch granules and soluble starch

B. circulans F-2 was reported earlier[7]. The strain cultivation was carried out essentially according to the method of our previous paper[8] on the medium containing crosslinked potato starch. Crosslinked starch was manufactured by crosslinking reaction between starches and epichlorohydrin [2].

Results

Proteolysis of BF-2A with subtilisin and proteinase K yielding identical protein fragments of 63 and 30 kDa

The RSDA of *B. circulans* F-2 has been separately incubated with proteolytic enzymes such as Asp-endopeptidase, Lys-endopeptidase, Arg-endopeptidase, and subtilisin. Of them, only subtilisin gave enzymatically active amylase band on the native-PAGE followed by zymobraphy. The chromatograms obtained during subtilisin digestion showed two increasing peaks and one decreasing peak, strongly suggesting that two predominant fragments are formed. After 11 hr digestion with subtilisin, the

chromatographic pattern remained constant. Two peptides were isolated using a preparative gel filtration on the TSK Gel G3000 SW-XL column. The results of SDS-PAGE clearly indicated that the original peptide was disappeared after subtilisin treatment and that a new peptide BF-2A' (63,000 Da) had appeared together with another smaller peptide BF-2B (30,000 Da). Activity staining showed that a larger peptide still had the amylase activity, but the smaller one had none.

Subtilisin cleavage of BF-2A is unique and provides important structural and functional information. BF-2A consists of two main structural parts: A catalytically active core and a raw starch binding region. BF-2A' is an incomplete enzyme lacking the binding domain. This is suggested by the fact that its adsorption rate onto raw starch granules is low. Catalytic cores obtained from BF-2A by subtilisin and proteinase K cleavage were fully active against soluble starch and maltooligosaccharides, although the activity towards raw starch was virtually absent. This suggests that a fragment (30 kDa) is initially split off in the BF-2A region and the existance of a region or domain of the enzyme that is sigificantly exposed, allowing access to the proteases. Hence, there is only one protease-sensitive domain for subtilisin and proteinase K.

Enzymatic properties of catalytic BF-2A'

The modified BF-2A' amylase exhibited maximum activity at 55 C, while the original amylase showed maximum activity at 59 C, indicating that thermal shift in the optimum temperature is possibly due to limited proteolysis. The enzyme BF-2A' was unstable at higher temperature, retaining about 40% of its original activity at 50 C. BF-2A' is more unstable than the BF-2A.

Original amylase, BF-2A is a G_6-forming amylase. In the early stage of hydrolysis, maltohexaose(G_6) was a main product detected and its amount increased rapidly and maltotetraose(G_4) and maltose(G_2) began to appear concomitantly after G_6 had accumulated to a certain degree. However, the BF-2A' produced a series of maltooligosaccharides from soluble starch. On further incubation, maltopentaose (G_5), G_4, maltotriose(G_3) and G_2 were formed with a concomitant decrease in G_9 and G_8. In final stage of hydrolysis, G_6 and G_7 accumulated with the disappearance of G_9 and G_8 having mobilities close to those of G_6 and G_7 on thin layer chromatography.

Raw and soluble starch-adsorbability and digestibility

The BF-2A was able to be effectively adsorbed onto insoluble polysaccharide of raw potato starch, but adsorbability of the BF-2A' onto raw starch decreased to about 17% of that the BF-2A. The digestibility of the BF-2A' decreased to about 18 % of the BF-2A. However, the digestibilities of the BF-2A' and BF-2A onto soluble starch were observed to be almost the same as those of the BF-2A. In K_m, Vmax and specific activity for hydrolysis of soluble starch, no differences in any of these parameters were detected for the two enzymes (Table 1).

RSDA contains a distinct domain structure

S. aureus V8 protease (endopeptidase Glu-C) cleaved the purified enzyme into fragments of 75, and 63 kDa. Small amounts of 60- and 50-kDa fragments were also observed. It is postulated that the proteolytic pathway for Glu-C involves the initial fragmentation of the 93-kDa original BF-2A into a major protein of 63-kDa via an intermidiate protein of 75-kDa. Intact BF-2A was also proteolysed with thermolysin and BF-2A disappeared with incubation, and new bands of A1 to A6 gradually appeared with increasing time. The proteolytic pathway of the enzyme was, therefore, estimated to be; 93-kDa

Table 1-1. Properties of the original and modified enzymes

Enzyme	Specific activity (units/mg of protein)[a]	Relative activity		Vmax (g/min/ml)[d]	K_m (%)[d]
		Soluble starch[b]	Raw starch[c]		
BF-2A	62.4	1.0	1.0	8.4	0.49
BF-2A'	64.7	0.97	0.16	7.8	0.48

[a]Specific activity against soluble starch for enzyme purified to homogeneity.
[b]Activities expressed relative to activity of the original enzyme (BF-2A) against soluble starch, which was designated as 1.0.
[c]Activities expressed relative to activity of the original enzyme against raw starch, which was designated as 1.0.
[d]Measured for the hydrolysis of soluble starch.

Table 1-2. Schematic comparison of proteolytic fragmentation of RSDA

Proteases	Starting molecule	Intermediate fragment	Major common fragment	Minor fragment
Subtilisin	93(BF-2A)		63(BF-2A'), 30(BF-2B)	
Proteinase K	93(BF-2A)		63(BF-2A'), 30(BF-2B)	
Glu-C	93(BF-2A)	75(A1)	63(A2)	60 or 50
Thermolysin	93(BF-2A)	75(A1)	63(A2) or 60(A3)	55(A4), 50(A5), 48(A6)

→75-kDa→63-kDa→60- or 55-kDa, plus minor A5 and A6. It was suggested that the inability of the protease-cleaved enzyme to hydrolyze starch granules is not due to an alteration of the molecular properties of the original enzyme. This indicates no global changes in the tertiary structure of the enzyme. The same subtilisin and proteinase K proteolysis cleavage patterns are observed for thermolysin-digests and for Glu-C-digests.

Results using truncated forms of rsda gene indicate the same distinct domain structure

Truncated forms of *rsda*, in which the gene sequence encoding the conserved domain had been deleted, directed the synthesis of a functional amylase that did not bind to raw starch. The functional role of the carboxyl-terminal sequences of the RSDA is investigated using clones encoding either the entire *rsda* or the truncated forms of *rsda*, which has the C-terminal sequences deleted. The comparison of the enzymatic properties of the the enzymes reveals that the C-terminal part of the enzyme is involved in the ability of this enzyme to bind raw starch. The conserved region of RSDA constitutes a raw starch-binding domain, which is distinct from the active centre. The possible role of this substrate-binding region will be discussed.

Discussion

A drastic change in the action pattern of amylase after subtilisin treatment was observed. This is a first report of a change in raw starch-digestibility of amylase and in action pattern of amylase after limited proteolysis. The most interesting results from this work are that hydrolytic activity of the original amylase from *B. circulans* F-2 against soluble starch is not affected by digestion with subtilisin, whereas raw-starch-digestibility rapidly decreased. Furthermore, loss in activity toward raw starch is paralleled by a decrease of enzyme adsorption onto this insoluble substrate, while the adsorbability of

the smaller peptide on raw substrate showed the same value as that of the original amylase. From the evidence presented it appears that subtilisin splits off the terminal region of the original enzyme corresponding to the carboxyl-termini, which has a lot of serine and threonine residues. This conclusion is dependent on the amino acid sequence data deduced from the cloned BF-2A gene

The stability of the modified amylase (BF-2A') activity on environmental conditions such as pH or thermal changes was compared with that of the original amylase (BF-2A). BF-2A' largely lost its stabilities on the above conditions. This result can support that the smaller peptide stabilizes the BF-2A in environment.

As similar works, it was reported that the raw starch-digestive glucoamylase I of *A. awamori* was digested with subtilisin or acid protease of the same mold strain [9] and the enzyme was converted to raw starch-indigestive glucoamylase I' and a glycopeptide. Crystalline cellulose-adsobable cellobiose oxidase from *P. chrysosporium* was also cleaved by papain into two domains, which one domain could be responsible for adsorption onto avicel, and the other could not be adsorbed onto the cellulose[10]. Sequence homology data has recently shown that similar domains are present in many granular-starch-digesting enzymes, including -amylase from *S. limosus*, -amylase from *C. thermosulfurogenes,* and cyclodextrin glucanotransferase from *K. pneumoniae* [10]. An analogous structure was reported for fungal cellobiohydrolases in which the catalytic domain and the binding domain were connected by a highly glycosylated region in serine, threonine and proline. Recent evidence suggests that this interconnecting region is not required for the binding domain to interact with insoluble substrates [11].

It was considered that raw starch-digestive amylase could be proteolysised by some proteolytic enzymes, and that carboxyl-terminal domain, consisting of the serine and threonine rich sequence, could be removed if it had a domain structure. A functional organisation of the original amylase is proposed; one domain, corresponding to the carboxyl-terminal of the enzyme is implicated in adsorption process, whereas the other domain contains hydrolytic, functional domain, corresponding to the amino-terminal region of the enzyme. The relative ease with which the terminal peptide is split off suggests that two parts of the enzyme molecules are themselves of structural domains, probably linked by some hinge region susceptible to proteolytic attack. Compared with other cases such as raw starch-digesting glucoamylases and crystalline cellulose-hydrolyzing cellulases, the most outstanding features of the BF-2A' would be that BF-2A' have a little activities of adsorption (about 17%) and digestion (about 18%) on raw starch, whereas no other cases showed these abilities at all to update and BF-2A has no any carbohydrate-moiety in the polypeptide. However, all the other cases indicated that glycosylated carboxyl-terminal region is responsible for adsorption onto raw substrates. The site is proposed as raw substrate-affinity site. Amino acid residues of the serine and threonine is closely related to function of raw substrate-affinity site. Thus, a raw starch adsorption site as distinct from hydrolytic site should be considered and this would be a first report indicating that non-glycosylated terminal region having the serine and threonine residues has a adsorbability onto raw substrate. The present results demonstrated that raw starch-digestibility of alpha-amylase from the bacterial strain was from its two distinct regions of a hydrolytic domain and a raw starch-adsorption domain.

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