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Subsite mapping of the binding region of α-amylases with a computer program

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A computer program has been evaluated for subsite map calculations of depolymerases. The program runs in WINDOWS and uses the experimentally determined bond cleavage frequencies (BCFs) for determination of the number of subsites, the position of the catalytic site and for calculation of subsite binding energies. The apparent free energy values were optimized by minimization of the differences of the measured and calculated BCF data. The program called SUMA (SUbsite Mapping of α-Amylases) is freely available for research and educational purposes via the Internet (E-mail: gyemant@tigris.klte.hu).

The advantages of this program are demonstrated through α-amylases of different origin, e.g. porcine pancreatic α-amylase (PPA) studied in our laboratory, in addition to barley and rice α-amylases published in the literature.

Results confirm the popular 'five subsite model' for PPA with three glycone and two aglycone binding sites. Calculations for barley α-amylase justify the '6 + 2 + (1) model' prediction. The binding area of barley α-amylase is composed of six glycone, two aglycone binding sites followed by a barrier subsite at the reducing end of the binding site. Calculations for rice α-amylase represent an entirely new map with a '(1) + 2 + 5 model', where '(1)' is a barrier subsite at the nonreducing end of the binding site and there are two glycone and five aglycone binding sites. The rice model may be reminiscent of the action of the bacterial maltogenic amylase, that is, suggesting an exo-mechanism for this enzyme.

Keywords: subsite mapping; α-amylase; action pattern; WINDOWS program.

X-ray crystallographic analysis, where the proteins in the crystalline state are free or complexed to a substrate-analogue, is a powerful method for mapping the active site of an α-amylase. However, these data can vary according to the crystalline varieties (free enzyme, enzyme-substrate/inhibitor complexes) or are not available at all. Therefore, the use of modified, low-molecular mass substrates could be an effective way to elucidate the number of subsites in the active site area of α-amylases.

In this study we have invoked the popular 'subsite model', which was introduced by Phillips [1], to account for the enzymatic properties of α-amylases such as PPA, barley and rice α-amylases.

The amylase subsite model [2] depicts the substrate binding region of the enzyme to be a tandem array of subsites. Each subsite is complementary to, and interacts with a substrate monomer unit. The subsites are labelled from the catalytic site, with negative numbers for subsites to the left (non reducing end side) and positive numbers to the right (reducing end side) according to the proposed nomenclature of Davies *et al.* [3]. There are a number of different ways in which an oligomer substrate can interact with these subsites. A substrate oligomer can bind nonproductively so that a susceptible bond does not extend over the catalytic amino acids of the enzyme; alternatively, the substrate can bind productively so that a susceptible bond lies over the catalytic site, in which case the bond is cleaved.

The process of quantifying the subsite model is referred to as subsite mapping. To completely map the binding region of α-amylases, we determined the number of subsites, located the position of the catalytic amino acids within the subsites and determined the binding energies of each subsite-substrate monomer unit. The method of subsite mapping originates from the early 1970s. Quantitative theories of the action pattern of amylase in terms of subsite affinities were proposed independently by Hiromi *et al.* [4] and Allen & Thoma [5] and later Suganuma *et al.* [6]. Hiromi proposed a kinetic method for evaluating the subsite affinities from the dependence of hydrolytic rate on the degree of polymerization (DP) of substrates, while Allen & Thoma developed a method based on the product analysis. Both methods have their advantages and disadvantages. The product analysis method of Allen & Thoma is suitable for endo-amylases, but can not be applied to exo-amylases. The kinetic method of Hiromi *et al.* is especially useful for exo-amylases, but its application to endo-amylases requires input from product analysis. The Suganuma method is based on the calculation of the kinetic parameter (k_0/K_m) and the BCF data at sufficiently low substrate concentration, where secondary attacks on the substrate can be ignored. Subsite maps for Taka-amylase A were evaluated by all three authors and quite similar subsite structures were

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Abbreviations: BCF, bond cleavage frequency; BLA, *Bacillus licheniformis* α-amylase; CNP, 2-chloro-4-nitrophenyl group; DP, degree of polymerization; Gn, maltooligosaccharide of *n* glucopyranoside units; pNP, 4-nitrophenyl group; PPA, porcine pancreatic α-amylase.

Enzymes: α-amylase (EC 3.2.1.1); porcine pancreatic α-amylase (AMYP_PIG); barley α-amylase (AMY2_HORVU); rice α-amylase.

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obtained [5,6]. The Thoma method was recently used for subsite mapping of endopolygalacturonases [7]. We have been studying the action pattern of endo-amylases by product analysis, therefore the procedure of Allen & Thoma [5] was applied for subsite mapping and our computer program was based on their theory.

Subsite mapping is simplified for exo-enzymes because there is only one productive binding mode for each substrate. However, endo-acting enzymes form more productive binding modes resulting in a complex product pattern. The relative rate of formation of each product is called bond cleavage frequency (BCF), which gives information about the subsite-binding energy. By using BCFs for a series of oligomeric substrates, it is possible to calculate the subsite binding energy for each subsite on the enzyme binding region, with the exception of the two subsites adjacent to the catalytic site which are occupied by all productive complexes. A detailed description of the relationships can be found in the works of Allen & Thoma [5].

For subsite map calculation the preferred procedure is that suggested by Allen & Thoma [5]:

(a) Establish experimental conditions where secondary reactions (transglycosylation, secondary attack) are insignificant.

(b) Use end-labelled substrates to determine quantitative BCF for chain lengths that are large enough to span the entire binding region.

(c) Examine bond cleavage frequencies to estimate the number of subsites and the position of the catalytic site.

(d) Apply a minimization process to test the differences of measured and calculated BCF data.

The present studies were aimed at developing a computer simulation of the α-amylase subsite model. By using a minimization routine, the computer program is capable of predicting a subsite map from experimental parameters.

Only a few subsite maps have been found in the literature and detailed knowledge about subsite architecture of these well studied enzymes is scarce. Therefore, we hope that our efforts meet a long felt need concerning subsite mapping.

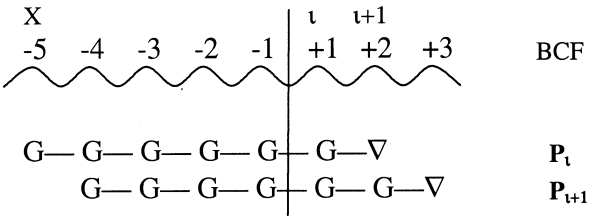
Subsite mapping has been evaluated for PPA, an α-amylase studied by us earlier. Also an attempt has been made to use this program for subsite mapping of other α-amylases found in the literature. Evaluations of subsite maps of rice and barley α-amylases are thus also presented.

MATERIALS AND METHODS

SUMA software: subsite mapping of amylases

This software calculates the apparent binding energies on the basis of the measured bond cleavage frequencies.

The calculations are based on the equation:



$$\Delta G_{l+1} - \Delta G_X = -RT \ln P/P_{l+1},$$

where $\Delta G_{ι+1}$ is the subsite binding energy of the subsite ι + 1, ΔG_X is the subsite binding energy of the subsite x, and $P_ι$ and $P_{ι+1}$ are the bond cleavage frequencies of the product which are produced from the binding mode in which the reducing end of the substrate are connected to subsite ι and ι + 1, respectively. Fig. 1 shows the structure of the program.

The supposed number of subsites and the position of the cleavage site can vary according to the calculations. The primary calculated subsite energy values can be refined to the best agreement of the measured and recalculated BCF data by the iteration. Fig. 2 shows the flow diagram of iteration. The graphical illustration of iteration appears in the 'Chart' window as a line chart (Fig. 3). The subsite energies are represented in 'Chart' window as subsite map (column or 3D-column chart) and are listed in 'Note'. The binding energies can be calculated and BCF data can be recalculated at temperatures other than those used for the measurement.

Advantages of SUMA

Unlimited input data possibilities. Simple usage. WINDOWS compatible, help in the menu. Note is used for saving, editing and printing calculated data. Graphical illustrations (minimization, subsite map) make the results clearer. BCFs can be calculated for substrates longer or shorter than those measured earlier.

SUMA is freely available for research and educational purposes (E-mail: gyemant@tigris.klte.hu).

Action patterns of α-amylases

Action patterns are summarized in the tables below: Table 1 contains our measurements resulting in the product ratios for PPA [8].

Tables 1, 2 and 3 show the ratio of products of PPA [9], barley [10] and rice [11] α-amylases found in the literature.

RESULTS AND DISCUSSION

The application of homologous oligomeric substrates is an effective way to explore the nature of the binding site and the process of catalysis for α-amylases. Although the overall structure and the tertiary folding of the polypeptide chains of different α-amylases have been determined [12], less is known about the differences in the action of α-amylases on the homologous maltooligosaccharide series and only a few subsite maps have been evaluated for α-amylases until now [5,6,13]. Our β-CNP-maltooligosaccharides have turned out to be good substrates for further studies of the action pattern and subsite mapping of PPA and BLA [8,14]. Compared with other substrate series so far reported, for example, maltooligosaccharides [15] or α-NP-maltooligosaccharides [10], the CNP-maltooligosaccharides, which are β-glycosides, are unique as their preparation and use in the mapping of the active centre of α-amylases were reported by our laboratory for the first time [8]. This β-linkage is stable and is not hydrolysed by α-amylases therefore, the products of hydrolysis are always β-glycosides.

Selection of these glycosides as substrates has been based on their size (DP 4–10) and good yields when synthesized from CDs [16] or via chemoenzymatic procedures [17].

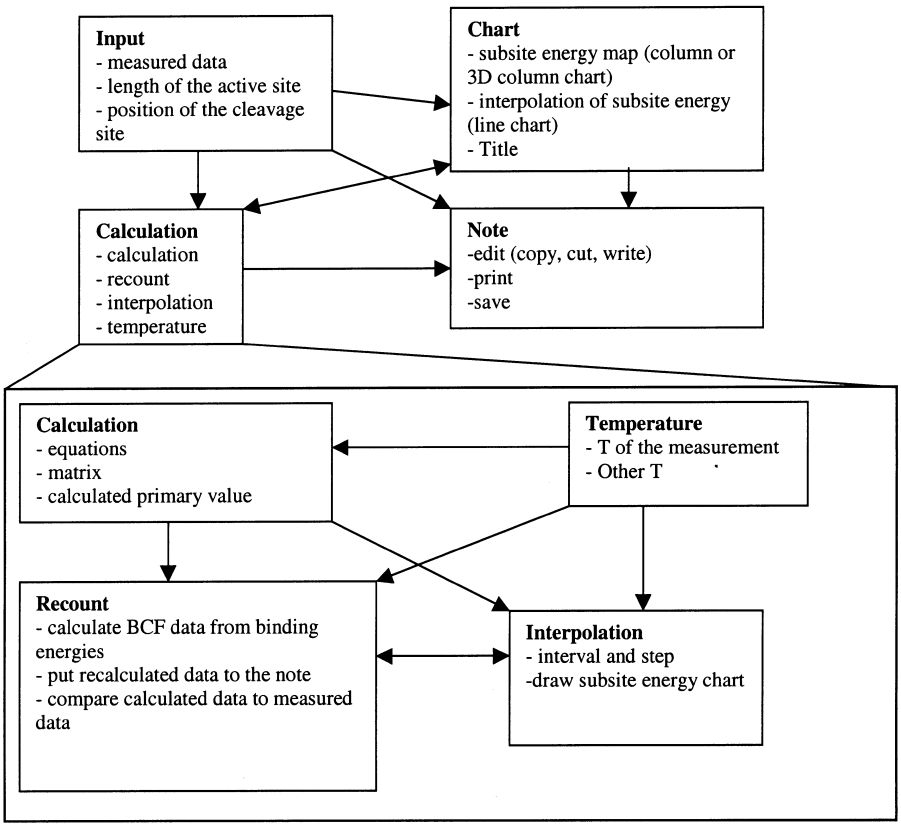


Fig. 1. Structure of the computer program.

Subsite mapping of PPA–'five subsite model' is confirmed

Porcine pancreatic α-amylase is one of the most exhaustively studied model enzymes of mammalian α-amylases [2,8,18,19]. A 'five subsite model' was suggested by Robyt and French [2] based upon kinetic studies of the action pattern of PPA on maltooligosaccharides of DP 4–8. Our findings were based on the action pattern of PPA on three different series of β-maltooligosaccharide glycosides and they confirmed the theory of five subsites [8]. However, the crystal structure of PPA isozyme II, in complex with the carbohydrate inhibitor acarbose, demonstrated the presence of six contiguous subsites for the binding of glucose units in the active centre of PPA [18].

In this study we made a subsite map evaluation by using the BCF data measured and published on CNP β-maltooligosides DP 4–8 [8]. Figure 4 shows the apparent energy of subsites confirming the 'five subsite model' of PPA.

A negligible apparent binding energy at subsite −4 (-0.8 kJ·mol^{-1}) might indicate a binding subsite, but this is not convincing at all.

The computer modeling on the two different maltooligosaccharide series resulted in the same subsite map for PPA. As it can be seen in Fig. 4, the calculated binding energies from BCF values on linear maltooligosaccharides DP 4–8 [9] are in very good agreement with the energy data calculated from BCF values on CNP β-maltooligosides DP 4–8. The negligible binding energy (-0.5 kJ·mol^{-1}) at subsite (−4) does not confirm the presence of an additional subsite.

Results confirm that the five subsites, originally assumed from experimental data, are correct and BCF are measured correctly within experimental error [8].

Finally, the two subsite maps, corresponding to each other, verify that the presence of the CNP at the reducing end of the substrates does not influence the apparent binding energies.

Subsite mapping of barley α-amylase isozyme 1 – justification of a '6 + 2' model

Action patterns of barley α-amylase isozymes 1 and 2 were published by MacGregor *et al.* [10] on maltooligosaccharides and their pNP α-glycosides of DP 4–7. These isozymes release not only pNP-containing products but also pNP from pNP-G_4, pNP-G_6 and pNP-G_7 substrates which are considered for the explanation of substrate bindings. The subsite affinities were not calculated in this work. The authors made proposals for the type and strength of interactions. A '7 + 3 model' was suggested for barley α-amylase isozyme 1, where the energy of interaction is favourable at subsites −6 and +2, less favourable at subsite −7 and unfavourable at subsite +3. They assume further unfavourable energy of interaction at subsite −5.

We made a calculation for BCF data using the published ratio of pNP-glycoside products, considering only the interactions between glucose units and subsites. Our subsite model proposed for barley α-amylase isozyme 1 (Fig. 5) partly confirms the suggestion by MacGregor *et al.* [10].

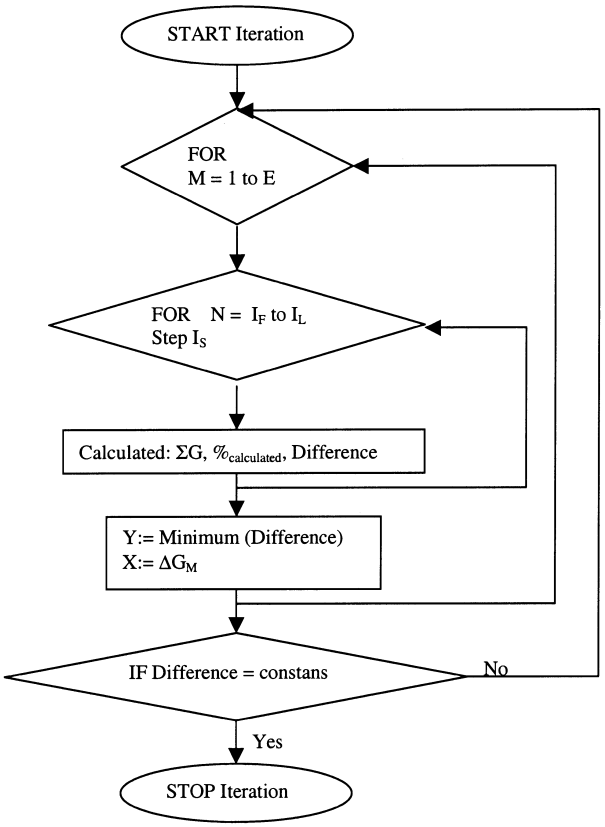


Fig. 2. Flow diagram of iteration. M and N, cycle variable; E, number of subsites; I_F, first iteration value; I_L, last iteration value; I_S, step iteration value; ΣG, the sum of the binding energies of the occupied sites; $\%_{calculated}$, the bond cleavage frequency calculated according to the subsite map; Difference, the sum of the difference between the measured and calculated data. During the iteration we look for the smallest possible value of 'Difference'; ΔG_M, the energy corresponding to the smallest 'Difference'.

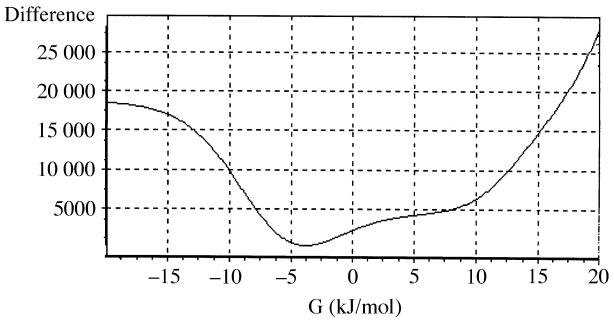


Fig. 3. Graphical illustration of iteration for −3 subsite of BLA. Apparent binding energy value (−5.1 kJ·mol^{-1}) can be found at the minimum of 'Difference'.

The calculated binding energy at subsite −6 (−12.2 kJ·mol^{-1}) indicates a remarkably good interaction with the monomer unit of the substrates compared with the other subsite energies. An unfavourable interaction (+2.7 kJ·mol^{-1}) could be found at subsite +3 suggesting the presence of a barrier subsite. Unlikely, the binding energy of the interaction was zero at subsite −7, and rather unfavourable at subsite −3 (+0.4 kJ·mol^{-1}) than at subsite −5 (−2.4 kJ·mol^{-1}). On the basis of these affinities, the total number of subsites for barley α-amylase is nine; six glycone binding sites and two aglycone binding sites followed by a barrier subsite.

The action of barley α-amylase on amylose as substrate was also studied earlier by MacGregor & MacGregor [20] and a '6 + 2 model' was proposed. Our calculations seem to justify this structure with an extra barrier subsite.

There is also another study on the action pattern of barley enzyme including calculation of subsite affinities which was based on the Suganuma method [21]. This calculation suggests a '6 + 4 model' for barley amylase, where the energy of interaction is very favourable at subsite −6. These results are in a good agreement with our calculations with the only exception relating to the +3 subsite. The group of Marchis–Mouren [21] found a low affinity at subsite +3, however, our calculations show a barrier site for the same subsite. The reason of this disagreement might be the absence of minimization or the relatively short substrates used.

There is a very interesting proposal of these authors; the active center might contain two parts, one comprising subsites −2, −1, +1, +2, +3, +4 and the second part comprising subsites −4, −5, −6. Our results are consistent with this proposal; the small positive binding energy at subsite −3 may be the border between the two parts.

Subsite mapping of rice isozyme Amy 3D – first subsite mapping, assuming a '2 + 5 model'

The action pattern of rice isozyme Amy 3D on pNP α-maltooligosides of DP 3–6 was published quite recently [11]. Amy 3D isozyme was expressed by *Saccharomyces cerevisiae* and produced substantial amounts of glucose from starch. No suggestion for the structure of the active site was given.

Our subsite model (Fig. 6) calculated for this rice α-amylase isozyme shows a very interesting and unusual profile. A barrier subsite exists at the nonreducing end of the binding site (+5.7 kJ·mol^{-1}) followed by two glycone and five aglycone binding sites. Interestingly, we found unfavourable energy of interaction (+2.7 kJ·mol^{-1}) at subsite +3 which was compensated by the high (−6.6 kJ·mol^{-1}) favourable energy of interaction at subsite +5.

Our study serves as the first characterization of the substrate binding site of rice isozyme Amy 3D. We describe the first subsite map with the calculated apparent binding energies. We suggest that the binding region of rice isozyme Amy 3D is composed of at least eight subsites; two glycone binding sites −2, −1 with an additional barrier subsite −3 and five aglycone binding sites. The presence of the barrier subsite at the end of the glycone binding site is suggesting an exo-mechanism for this enzyme. The action pattern also indicates the exo-mechanism, because the glucose or maltose as main products are released from the non reducing end of each substrate.

CONCLUSIONS

The present paper describes a method, developed for the quantitative determination of subsite maps of α-amylases. Complete subsite maps have been evaluated by using the experimentally determined BCFs for the characterization of the binding region of PPA [8] and BLA [14]. The product

Table 1. BCFs of PPA [8,9]. Hydrolysis conditions for CNP-glycoside products: 0.5 mM substrate, 50 mM Hepes buffer (pH: 6.9), 37 °C. Hydrolysis conditions for non-CNP products: 0.5 mM substrate, 50 mM sodium phosphate buffer (pH: 6.8), 30 °C.

	Products (mol% of CNP-glycoside products) [8]					Products (mol% of products) [9]				
Substrate	G_1-CNP	G_2-CNP	G_3-CNP	G_4-CNP	G_5-CNP	G_1	G_2	G_3	G_4	G_5
G_4(-CNP)	38	62				36	64			
G_5(-CNP)		100					100			
G_6(-CNP)		54	46				54	46		
G_7(-CNP)		44	34	22			41	33	26	
G_8(-CNP)		25	40	25	10		28	37	24	11

Table 2. BCFs of barley α-amylase isozyme [10]. Hydrolysis conditions: 5 mg mL^{-1} substrate, 0.1 M acetate buffer (pH: 5.5), 35 °C.

	Products (mol% of NP-glycoside products)				
Substrate	G_1-pNP	G_2-pNP	G_3-pNP	G_4-pNP	G_5-pNP
G_4-pNP	24	63	13		
G_5-pNP	40	44	13	2	
G_6-pNP	42	26	12	10	10
G_7-pNP	98	2			

Table 3. BCFs of rice α-amylase Amy3D [11]. Hydrolysis conditions: 4 mM substrate, 50 mM sodium acetate buffer (pH: 5.5), 30 °C.

	Products (mol% of NP-glycoside products)				
Substrate	G_1-pNP	G_2-pNP	G_3-pNP	G_4-pNP	G_5-pNP
G_3-pNP	56	44			
G_4-pNP	9	88	3		
G_5-pNP	10	19	60	11	
G_6-pNP				28	72

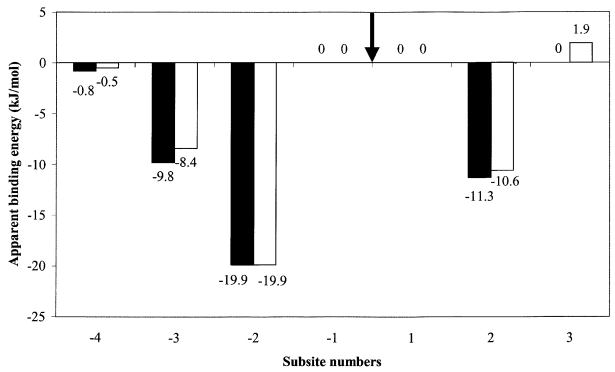


Fig. 4. Subsite maps for porcine pancreatic α-amylase (PPA). The solid bars are related to CNP-modified maltooligosaccharide substrates [8] and the open bars depict the subsite map with linear maltooligosaccharides [9]. The apparent binding energies were calculated according to the data of Table 1. The arrow indicates the location of hydrolysis. The reducing end of maltooligomers situated at the right hand of the subsite map. Negative energy values indicate bindings between the enzyme and aligned glucopyranosyl residues, while positive values indicate repulsion.

patterns have been determined by HPLC utilizing a homologous series of CNP-substituted maltooligosaccharides of DP 4–10 as model substrates.

Simultaneously, a computer program has also been developed using a minimization routine to establish a subsite map for PPA and BLA.

End-labelled substrates with chain lengths large enough to span the entire binding region of PPA and BLA met the requirements of getting the best subsite map. The results confirm that the nine subsites for BLA and the five subsites for PPA, originally assumed from our experimental data, are correct and bond-cleavage frequencies are predicted correctly.

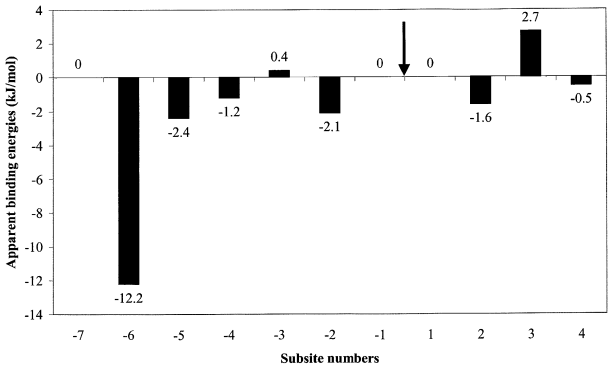


Fig. 5. Subsite map of barley α-amylase isoenzyme. The binding affinities were calculated according to the data of Table 2.

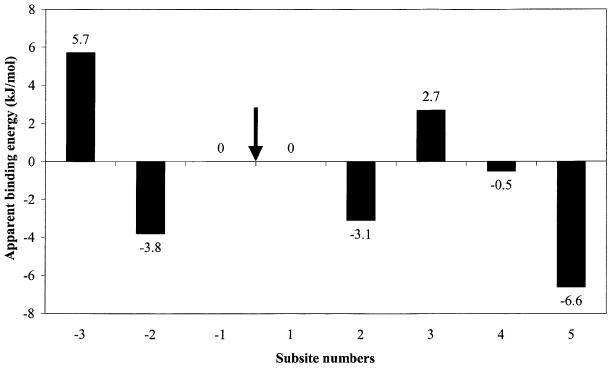


Fig. 6. Subsite map of rice α-amylase isoenzyme (AMY3D). The binding affinities were calculated according to the data of Table 3.

We also show how this computer program can be applied to BCF data accessible in the literature to ascertain the number of subsites and establish the binding energies of subsite-substrate monomer units of different α-amylases. However, if the substrates are not long enough, as we found for barley and rice amylases DP 4–7, DP 3–6, respectively, the results should be interpret with care. To confirm the subsite maps of barley and rice amylases, it is necessary to re-examine BCFs for substrates having a longer chain lengths than that of the binding site.

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