Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent.

Francisco J. Plou a, M. Angeles Cruces a, Manuel Ferrer a, Gloria Fuentes a, Eitel Pastor a, Manuel Bernabé b, Morten Christensen c, Francisco Comelles d, José L. Parra d and Antonio Ballesteros a

a Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Cantoblanco, 28049 Madrid, Spain.
b Instituto de Química Orgánica, CSIC, 28006 Madrid, Spain.
c Novozymes A/S, Novó Allé, 2880 Bagsvaerd, Denmark.
d Instituto de Investigigaciones Químicas y Ambientales de Barcelona, CSIC, 08034 Barcelona, Spain.

Corresponding author: Francisco J. Plou, Departamento de Biocatálisis, Instituto de Catálisis, CSIC, Campus UAM Cantoblanco, 28049 Madrid, Spain. Phone: 34-91-5854869; Fax: 34-91-5854760. E-mail: fplou@icp.csic.es
ABSTRACT

Enzymatic synthesis of fatty acid esters of di- and trisaccharides is limited by the fact that most biological catalysts are inactivated by the polar solvents (e.g. dimethylsulfoxide, dimethylformamide) where these carbohydrates are soluble. This article reviews the methodologies developed to overcome this limitation, namely those involving control over the reaction medium, the enzyme and the support. We have proposed the use of mixtures of miscible solvents (e.g. dimethylsulfoxide and 2-methyl-2-butanol) as a general strategy to acylate enzymatically hydrophilic substrates. We observed that decreasing the hydrophobicity of the medium (i.e. lowering the percentage of DMSO) the molar ratio sucrose diesters vs. sucrose monoesters can be substantially enhanced. The different regioselectivity exhibited by several lipases and proteases makes feasible to synthesize different positional isomers, whose properties may vary considerably. In particular, the lipase from Thermomyces lanuginosus displays a notable selectivity for only one hydroxyl group in the acylation of sucrose, maltose, leucrose and maltotriose, compared with lipase from Candida antarctica. We have examined three immobilisation methods (adsorption on polypropylene, covalent coupling to Eupergit C, and silica-granulation) for sucrose acylation catalyzed by T. lanuginosus lipase. The morphology of the support affected significantly the reaction rate and/or the selectivity of the process.
INTRODUCTION

Sugar esters are non-ionic surfactants that consist of a carbohydrate moiety as hydrophilic group and one or more fatty acids as lipophilic component. By controlling the esterification degree and the nature of fatty acid and sugar, it is possible to synthesize sugar esters within a wide range of hydrophilic-hydrophobic balance (HLB) and, in consequence, of properties.

Sugar fatty acid esters have broad applications in the food industry (Nakamura, 1997; Watanabe, 1999). Other fields of application include cosmetics, detergents, oral-care products and medical supplies. In addition, their properties as antibiotics (Marshall and Bullerman, 1994), antitumorals (Okabe et al., 1999) and insecticidals (Chortyk et al., 1996) are well reported and might open new markets. The current production of sucrose esters, by far the most developed derivatives of this group, is estimated to be about 4000 Tm per year (Hill and Rhode, 1999).

Regioselective acylation of carbohydrates is an arduous task due to their multifunctionality (Descotes et al., 1996). Sugar esters can be synthesized using either chemical and/or enzymatic processes. Current chemical production of sucrose esters is usually base-catalyzed at high temperatures, has a low selectivity, forming coloured derivatives as side-products (Nakamura, 1997). Selective chemical acylation, however, requires complex protecting-groups methodologies (Vlahov et al., 1997).

Enzymes have been successfully applied to the regioselective transformations of mono- and oligosaccharides, including acylation, deacylation and oxidation reactions. The enzyme-catalyzed synthesis of sugar esters provides regio- and stereoselective products (Cruces et al., 1992; Riva et al., 1998; Soedjak and Spradlin, 1994).

In this paper we will review the main parameters that need to be considered for the mono- and diacylation of sugars, focusing on di- and oligosaccharides.
INFLUENCE OF THE REACTION MEDIUM

Methodologies for sugar acylation need to find a medium where a polar reagent (the carbohydrate) and an apolar reagent (the fatty acid donor) are able to react in presence of the biocatalyst.

Two main strategies have been developed to overcome this particular limitation. The first is based on the use of organic solvents suitable for the solubilization of both the saccharide and the acylating agent (Plou et al., 1995; Rich et al., 1995; Riva et al., 1988; Soedjak and Spradlin, 1994). The second is based on the hydrophobization of the sugar moiety by different methods: complexation with phenylboronic acids (Ikeda and Klibanov, 1993), formation of acetals (Sarney et al., 1994) or chemical acetylation (Steverink-de-Zoete et al., 1999). The derivatization is followed by solvent-free esterification with fatty acids. However, we will focus on single-step acylations in organic solvents, since multi-step processes are less probable to attract the interest of industries.

Although some proteases catalyze the acylation of several di- and oligosaccharides in solvents such as DMF and pyridine (Table 1), most enzymes are readily inactivated by polar solvents capable of dissolving di- and trisaccharides to a large extent –dimethylsulfoxide (DMSO), dimethylformamide (DMF), dimethylacetamide (DMA), etc. –.

In order to avoid the use of these solvents, and at the same time to exploit the use of lipases in these reactions, several processes have been recently reported using more benign solvents such as tertiary alcohols or ketones, that dissolve the carbohydrate only partially. This strategy has been very fruitful for monosaccharides, e.g. the acylation of glucose and fructose has been successfully achieved in solvents such as tert-butyl alcohol (Degn et al., 1999), 2-methyl-2-butanol (Chamouleau et al.,
2001) or acetone (Arcos et al., 1998). However, the comparatively lower solubility of di- and oligosaccharides in these solvents makes difficult to get notable yields (Table 1).

In this context, we developed a lipase-catalyzed process using a medium constituted by two miscible solvents. More specifically, the sugar was dissolved in a low amount of a hydrophilic solvent (basically dimethylsulfoxide), and then was added to a tertiary alcohol, namely 2-methyl-2-butanol. This increased substantially the solubility of the carbohydrate, thus allowing the acylation to proceed. As the reaction medium is mostly composed of 2-methyl-2-butanol (where most lipases are significantly stable), the inactivation of the biocatalyst is greatly reduced.

We applied this methodology to the acylation of sucrose, maltose, leucrose and maltotriose, with fatty acids ranging from 8 to 18 carbon atoms. With all the di- and trisaccharides tested, the pre-dissolution of the sugar in DMSO caused a notable acceleration of the reaction (Ferrer et al., 1999a, 2000a).

In enzyme-catalyzed reactions, the possibility of manipulating not only the reactivity, but also the selectivity, by the proper choice of the solvent, is of great interest. For this reason, the effect of DMSO percentage on the kinetics of the reaction using the lipase from *Thermomyces lanuginosus*, immobilized in Celite by precipitation, was studied (Fig. 1). When 5% DMSO was present in the final reaction mixture, the formation of diesters was very significant, especially using sucrose as substrate. In contrast, when the reaction was carried out in a medium containing 20% DMSO, the acylation is much slower but, interestingly, the presence of diesters was almost negligible (< 1%). This allows to control the selective production of monoester or diester, simply modifying the percentage of DMSO. Our results seem to indicate that diester formation is higher when increasing solvent hydrophobicity.

At DMSO percentages higher than 30%, the reaction rate was negligible, probably due to the inactivation of the lipase by DMSO, resulting in a disruption of the

---

*Journal of Biotechnology, 96, 55-66 (2002)*
hydration shell on the lipase surface (Matsumoto et al., 1997). It is well known that DMSO can also cause unfolding of proteins (Klyosov et al., 1975).

The effect of solvent nature on the activity and stability of biocatalysts in organic media has been widely investigated (Rich et al., 1995; Almarsson & Klibanov, 1996). Selection of the appropriate solvent for a reaction, with enzyme being active and stable, is always a critical point (Mozhaev, 1998; Tyagi and Gupta, 1998).
INFLUENCE OF THE ENZYME

For the enzyme-catalyzed transesterification of sugars, different regioisomers may be obtained with an appropriate election of the biocatalyst. This is remarkable because, for example, the properties of different sugar monoesters have been reported to vary significantly (Husband et al., 1998).

We screened different lipases and proteases for the acylation of sucrose and maltose with vinyl laurate in a mixture of 2-methyl-2-butanol and DMSO 4:1 (v/v). Among a battery of biocatalysts assayed, at least 4 lipases were capable to catalyze of these processes (Fig. 2). The lipase from Thermomyces lanuginosus was the most efficient enzyme for these reactions. The resulting monoesters were isolated.

In the case of sucrose, 1H-NMR analysis showed that hydroxyl 6-OH at the glucose ring had been selectively acylated. The lipase from Candida antarctica also yielded a significant sucrose conversion, but two monoesters (6- and 6′-) were formed in a nearly equimolar ratio. Lipase from Pseudomonas sp. immobilized on Toyonite and a new lipase isolated in our laboratory from the liquid wastes of Penicillium chrysogenum cultures (Ferrer et al., 2000b) were also notably active, yielding the 6-monoester.

The chemical reactivity of sucrose hydroxy groups follows the order, under most experimental conditions, 6-OH ≥ 6′-OH > 1′-OH > secondary-OHs (Descotes et al., 1999). Concerning reactivity towards enzymes, several proteases of the subtilisin family catalyze selectively the acylation of the primary 1′-OH at the fructose ring (Fig. 3). The lipases from Pseudomonas sp., Mucor miehei and Thermomyces lanuginosus are regiospecific for the hydroxyl 6-OH. Using the lipase from Candida antarctica, the reaction of sucrose with ethyl laurate gives rise to a mixture of 6- and 6′-monoesters (Woudenberg et al., 1996).
In the case of maltose, there exists a good correlation between chemical and enzymatic selectivity. In all experiments, the 6′-OH position of maltose is more reactive than the 6-OH at the reducing end.

Leucrose, an isomer of sucrose with α(1\(\rightarrow\)5)-glucosyl-fructose link, was also tested under similar conditions. The conversion to monoester was 82% in 8 h using 20% DMSO. The acylation of leucrose gave rise to 3 monoesters, although 6-O-lauroyl-leucrose represented 92% of the total.

When studying the acylation of the trisaccharide maltotriose, the hydroxyl 6\(''\)-OH in the non-reducing end was selectively acylated. Using 5% DMSO in the medium, a maltotriose conversion of 88% to monoester and 10% to diesters was obtained.
INFLUENCE OF THE ACYL DONOR

For lipase-catalyzed acylations, reactions take place via the formation of an acyl-enzyme intermediate (Kawase et al., 1992; Schmid & Verger, 1998). As a consequence, the nature of the acyl donor (both the fatty acid and the leaving group) has a notable effect on reactivity.

As shown in Table 1, most sugar acylations using proteases as biocatalysts have been carried out with short and medium chain fatty acids. We observed a lowering of the reaction rate when moving from butyrate to octanoate on the acylation of sucrose with trichloroethyl esters (Plou et al., 1995). For the acylation of sugars with long fatty acids, the diversity and nature of lipases convert them as the most promising catalysts. In fact, we studied the effect of fatty acid length (12-18 carbon atoms) on the kinetics of maltose acylation in the presence of the lipase from Thermomyces lanuginosus (Ferrer et al., 2000a). We observed that, except for vinyl stearate, the longer the carbon chain, the higher the conversion.

Wang et al. (1988) demonstrated that the rate of transesterification of hydroxyl–containing compounds (including sugars) with vinyl esters is about 20-100 times faster than with alkyl esters (the vinyl alcohol formed during the process tautomerizes to the low-boiling-point acetaldehyde, shifting the equilibrium towards the ester formation). However, one must be cautious since it has been reported recently that several lipases (e.g. from Candida rugosa and Geotrichum candidum) lose most of their activity when exposed to acetaldehyde (Weber et al., 1995). Apart from enol esters, the acylation of carbohydrates has been performed using trihaloethyl esters (Cruces et al., 1992), alkyl esters (Woudenberg et al., 1996), acid anhydrides (Uemura et al., 1989), oxime esters (Pulido and Gotor, 1992) and even free fatty acids (Ku and Hang, 1995).
EFFECT OF THE SUPPORT

Immobilization is a suitable approach to facilitate the substrates to reach the catalytic site of enzymes, minimizing protein-protein contacts that are present when using enzymes suspended in organic solvents. Immobilization allows easy separation and reuse of the biocatalyst, makes product recovery easier and is able to enhance resistance against inactivation by different denaturants (Tischer and Kasche, 1999). Several parameters of immobilized lipases are important to consider for industrial applications: mechanical strength, chemical and physical stability, hydrophobic/hydrophilic character, enzyme loading capacity, and cost.

For the acylation of sucrose with activated esters, a significant improvement of the reaction rate by immobilization of the enzyme has been reported (Cruces et al., 1992; Plou et al., 1995; Ward et al., 1997). In fact, most of the enzymatic acylations of sugars reported in the literature have been performed using immobilized commercial lipases.

We compared, under the same reaction conditions, the lipase from Thermomyces lanuginosus immobilized under three different methods: adsorption on polypropylene (Accurel EP100), covalent attachment to Eupergit C and silica-granulation (Ferrer et al., 2001). Eupergit C immobilized lipase showed a very low synthetic activity. The lipase adsorbed on Accurel showed an extraordinary initial activity, and after 30 min of reaction the formation of 6-O-monoester decreased owing to its transformation into diesters (6,6’- and 6,1’-) (Fig. 4). The highest selectivity to sucrose 6-monolaurate was found with granulated lipase. These differences in reactivity and/or selectivity might be related with the morphologies (average pore diameter, surface area, etc.) of the supports examined (see electron micrographs of Fig. 5).
Silica-based granulation is a new immobilization technique that makes use of inexpensive small-size silica to get granules of 300-1000 μm particle size and high enzymatic efficiency (Christensen et al., 1998). The granules exhibit high-pressure resistance, good filtrability and therefore are suitable for both batch and fixed-bed reactors (Pedersen and Christensen, 2000). The hydrophobic character of the granules may be modulated varying the source of silica.
EFFECT OF SUGAR ESTER STRUCTURE ON FUNCTIONAL PROPERTIES

Surface-active compounds synthesized from renewable resources, such as fatty acids and polyols, have increasing interest due to advantages with regard to performance, consumers health and environmental compatibility compared to petrol-derived standard products.

As shown before, careful control over the reaction medium, the enzyme and the support allows to drive the reaction towards the formation of a desired regioisomer. It is also possible to modulate the molar ratio monoester/higher esters.

The effect of acylation position and acylation degree on surfactant properties of sucrose-based esters has been hardly studied (Husband et al., 1998). In contrast with numerous works on properties of monosaccharide-based (Ducret et al., 1996; Scheckerman et al., 1995) and sucrose-based (Abran et al., 1989; Bazin et al., 1998) surfactants, few data is available characterizing other di- and trisaccharide esters, probably due to the lack of appropriate synthetic methods.

Depending on the molecular structure of the saccharide moiety (mono-, di- and trisaccharide), as well as the acyl chain length of the fatty acid, it is possible to get surfactants with different physicochemical characteristics, evidenced by the broad range of CMC (critical micellar concentration) values indicated in Table 2.

Our enzymatically-synthesized disaccharide esters showed CMC and surface tension values (Table 2) within the range of related compounds. Although similar values of surface tension are obtained, the main advantage of di- and trisaccharide esters with respect to the monosaccharides derivatives lies in their notably higher solubility in water, as a consequence of the increased hydrophilicity of the sugar head group. This confirms the interest of investigations for developing selective methods for
the preparation of di- and trisaccharide fatty acid esters.

Other properties that can be significantly affected by the structure of the sugar esters are those related with their antibiotic, insecticidal and antitumoral activities. In this context, we are currently assaying the antimicrobial activities of some of our derivatives against a series of microorganisms (Gram-positive, Gram-negative and yeasts, data not shown).
ACKNOWLEDGEMENTS

We are grateful to Loreto Bajón (Instituto de Catálisis) for technical help with electron microscopy. We are indebted to Jordi Sucrana (Degusa Texturat Systems, Barcelona, Spain) and Naoya Otomo (Mitsubishi Kagaku Foods Co., Tokyo, Japan) for technical help. We thank Comunidad de Madrid and Ministerio de Ciencia y Tecnología for research fellowships. This work was supported by the E.U. (Project BIO4-CT98-0363), the Spanish CICYT (Projects BIO98-0793 and BIO1999-1710-CE), and Comunidad de Madrid (Project 07G/0042/2000).
REFERENCES


Table 1. Examples of solvents employed for the enzymatic acylation of di- and trisaccharides with fatty acids.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Sugar solubility a</th>
<th>Carbohydrate</th>
<th>Acyl donor</th>
<th>Biocatalyst</th>
<th>T (°C)</th>
<th>Conversion (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>++</td>
<td>Sucrose</td>
<td>C8</td>
<td>Protease N</td>
<td>45</td>
<td>29 (2 days)</td>
<td>Carrea et al., 1989</td>
</tr>
<tr>
<td>Pyridine</td>
<td>+</td>
<td>Sucrose</td>
<td>C4-C12</td>
<td>Alcalase</td>
<td>45</td>
<td>49 (7 days)</td>
<td>Soedjak and Spradlin, 1994</td>
</tr>
<tr>
<td>Pyridine</td>
<td>+</td>
<td>Sucrose</td>
<td>C12-C18</td>
<td>Subtilisin (Chiro CLEC-BL)</td>
<td>40</td>
<td>80-90 (3 days)</td>
<td>Polat et al., 1997</td>
</tr>
<tr>
<td>DMF</td>
<td>++</td>
<td>Maltulose, Palatinose, Raffinose, Stachyose</td>
<td>C4</td>
<td>Subtilisin</td>
<td>30-45</td>
<td>&gt; 50 (9-24 h)</td>
<td>Riva et al., 1998</td>
</tr>
<tr>
<td>tert-Butanol</td>
<td>–</td>
<td>Sucrose, Maltose, Palatinose, Trehalose, Leucrose</td>
<td>C4-C12</td>
<td>C. antarctica lipase (reflux)</td>
<td>82</td>
<td>10-85 (24 h)</td>
<td>Woudenberg et al., 1996</td>
</tr>
<tr>
<td>tert-Butanol</td>
<td>–</td>
<td>Sucrose</td>
<td>Linoleic acid</td>
<td>B. fulva lipase</td>
<td>30</td>
<td>36.6 (24 h)</td>
<td>Ku and Hang, 1995</td>
</tr>
</tbody>
</table>

a Sucrose solubility at 30°C: ++ very soluble (>150 g/l); + moderate soluble (30-150 g/l); - little soluble (< 1 g/l), according to Kononenko and Herstein (1956)
Table 2. Surfactant properties of several mono- and disaccharide fatty acid monoesters.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Fatty acid</th>
<th>CMC (µM)</th>
<th>Surface tension (mN/m)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>C12–C16</td>
<td>250-28</td>
<td>31.5-35.2</td>
<td>Ferrer, 1999b</td>
</tr>
<tr>
<td>Maltose</td>
<td>C12-C16</td>
<td>240-6</td>
<td>35.8-39.0</td>
<td>Ferrer, 1999b</td>
</tr>
<tr>
<td>Lactose</td>
<td>C14–C16</td>
<td>43 – 11</td>
<td>38.6–39.5</td>
<td>Garafalakis et al., 2000</td>
</tr>
<tr>
<td>Glucose</td>
<td>C8–C12</td>
<td>2⋅10⁴ – 150</td>
<td>27.3–30.5</td>
<td>Ducret et al., 1996</td>
</tr>
<tr>
<td>Galactose</td>
<td>C14–C18:1</td>
<td>150–20</td>
<td>31–43</td>
<td>Garafalakis et al., 2000</td>
</tr>
<tr>
<td>Fructose</td>
<td>C10–C16</td>
<td>2⋅10³ – 30</td>
<td>27</td>
<td>Scheckerman et al, 1995</td>
</tr>
</tbody>
</table>
Legends to Figures

**Fig. 1.** Kinetics of acylation of sucrose and maltose with vinyl laurate varying the percentage of DMSO in 2-methyl-2-butanol. The conversions to monolaurate (λ) and dilaurate (O) are shown. The biocatalyst was in both cases the lipase from *Thermomyces lanuginosus* immobilized on Celite (12.5 ml of Lipolase 100L per g support). Conditions for sucrose acylation: 0.03 M sucrose, 0.3 M vinyl laurate, 50 mg ml\(^{-1}\) biocatalyst, 50 mg ml\(^{-1}\) molecular sieves (3 Å), 40°C. Conditions for maltose acylation: 0.12 M maltose, 0.3 M vinyl laurate, 25 mg ml\(^{-1}\) biocatalyst, 25 mg ml\(^{-1}\) molecular sieves (3 Å), 40°C. The progress of the reactions was followed by HPLC using a system equipped with a Spectra-Physics pump, a Nucleosil 100-C18 column (250 x 4.6 mm), and a refraction-index detector (Spectra-Physics). Methanol:water 85:15 (v/v) was used as mobile phase at 1.5 ml/min. The temperature of the column was kept constant at 40°C.

**Fig. 2.** Screening of immobilized lipases for the acylation of sucrose and maltose in 2-methyl-2-butanol:DMSO (4:1 v/v). The nature of the support is also indicated. Experimental conditions for sucrose acylation are described in Ferrer *et al.* (1999a), and for maltose acylation in Ferrer *et al.* (2000a).

**Fig. 3.** Reported regioselectivity by lipases and proteases in the acylation of several di- and trisaccharides.

**Fig. 4.** Kinetics of the acylation of sucrose with vinyl laurate using lipase from *T. lanuginosus* immobilized by: (A) Adsorption on Accurel (B) Granulation with Sipernat 22. Immobilization conditions are described in Ferrer *et al.* (2002). Reaction conditions: 0.03 M sucrose, 0.3 M vinyl laurate, 100 mg ml\(^{-1}\) biocatalyst, 100 mg ml\(^{-1}\) molecular sieves (3 Å), 40°C. Reaction medium was
2-methyl-2-butanol:DMSO (4:1 v/v). The conversion is expressed as the percentage of the initial sucrose converted into monoester and diester, as determined by HPLC (conditions of Fig. 1).

Fig. 5. Scanning electron micrographs of the supports used for immobilization of *Thermomyces lanuginosus* lipase.
Fig. 1
Fig. 2

Conversion to monolaurate in 24 h (%)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Sucrose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. lanuginosus Celite</td>
<td>51</td>
<td>47</td>
</tr>
<tr>
<td>C. antarctica Lewatit (Novozyme 435)</td>
<td>45</td>
<td>39</td>
</tr>
<tr>
<td>Pseudomonas sp. P. chrysogenum Toyonite (Lipase PS)</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>Celite</td>
<td>28</td>
<td>14</td>
</tr>
</tbody>
</table>
Sucrose

C. antarctica lipase (Woudenberg et al., 1996)

Subtilisin (Riva et al., 1988; Cruces et al., 1992; Soedjak & Spradlin, 1994; Rich et al., 1995; Polat et al., 1997)
Protease N (Carrea et al., 1989)

Maltose

Subtilisin (Riva et al., 1988)
B. fulva lipase (Ku et al., 1995)
C. antarctica lipase (Woudenberg et al., 1996)
T. lanuginosus lipase (Ferrer et al., 2000a)

Leucrose

T. lanuginosus lipase (Ferrer et al., 2000a)
C. antarctica lipase (Woudenberg et al., 1996)

Maltotriose

Subtilisin (Riva et al., 1988)
T. lanuginosus lipase (Ferrer et al., 2000a)
Fig. 4

**POLYPROPYLENE (Accurel EP100)**

Sucrose conversion (%)

**SILICA GRANULATE**

Sucrose conversion (%)

Time (h)
<table>
<thead>
<tr>
<th>Material</th>
<th>Magnification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCUREL EP-100</td>
<td>x 40</td>
</tr>
<tr>
<td>SILICA-GRANULATE</td>
<td>x 60</td>
</tr>
<tr>
<td>EUPERGIT C</td>
<td>x 60</td>
</tr>
<tr>
<td></td>
<td>x 8000</td>
</tr>
<tr>
<td></td>
<td>x 8000</td>
</tr>
<tr>
<td></td>
<td>x 8000</td>
</tr>
</tbody>
</table>